

Validation of Tools for Verification of Elimination of Transmission



PCR & Xenomonitoring Results from Belém & Maceió

Eliana Rocha, Gilberto Fontes, and Wendell A. P. Almeida,
Center of Biological Sciences, University of Alagoas
(Universidade Federal de Alagoas; UFAL) / Maceió; Reinaldo
Braun and Leôncio N. Pimentel, Municipal Secretariat of Health,
Belém; John Ehrenberg and Steve Ault, PAHO/WHO

Overview

In this study, conducted in Brazil by the UFAL, xenomonitoring was used to verify the natural infection of vectors. Results are presented from work done in Belém by staff from the Secretariat of Health, led by Dr. Reinaldo Braun.

PHASE I

Standardization

There was no standardized assay for either the extraction of deoxyribonucleic acid (DNA) from the existing parasites in the mosquitoes via polymerase chain reaction (PCR) or the determination of the optimum size of the mosquito pool size (the number of mosquitoes examined), so the initial phase of the research focused on standardizing these steps. Additional experiments were conducted to try to standardize the laboratory technique (based on conditions in Maceió) by varying the amount of primer, the number of cycles of amplification, and the concentration of the different reagents used in PCR.

PCR (Ssp1¹ sequence)

- DNA extraction from
 - *W. bancrofti* microfilaria (mf) isolated from human blood
 - *W. bancrofti* mf from experimentally infected mosquitoes
 - Non-infected mosquitoes + *W. bancrofti* mf
 - *Dirofilaria immitis* mf
 - Supplies
 - Promega extraction kit
 - Method
 - Phenol-chloroform method
 - Boiling method (Vasuki et al., 2003)
 - Other techniques described in the literature (Williams et al., 2002)

The PCR technique used by most laboratories, the Ssp1 sequence (which detects 188 base pairs, as described in the literature) was used to verify vector infection via DNA extraction from (1) *W. bancrofti* mf in human blood, (2) laboratory-bred *W. bancrofti* mf in experimentally infected mosquitoes, (3) non-infected mosquitoes + *W. bancrofti* mf, and (4) *Dirofilaria immitis*, another mf that

¹ Protein of *Schizosaccharomyces pombe* (fission yeast) involved in actin localization and thus in polarized cell growth.

exists in Brazil and has the same vector as *W. bancrofti* (the *Culex quinquefasciatus* [Cq] mosquito). A Promega DNA extraction kit was used to apply the phenol-chloroform technique, the boiling technique (Vasuki et al., 2003), and other techniques described in the literature (e.g., Williams et al., 2002). Figure 1 shows the results of the use of the phenol-chloroform method (the gold standard for the study) compared to those produced by the boiling method.

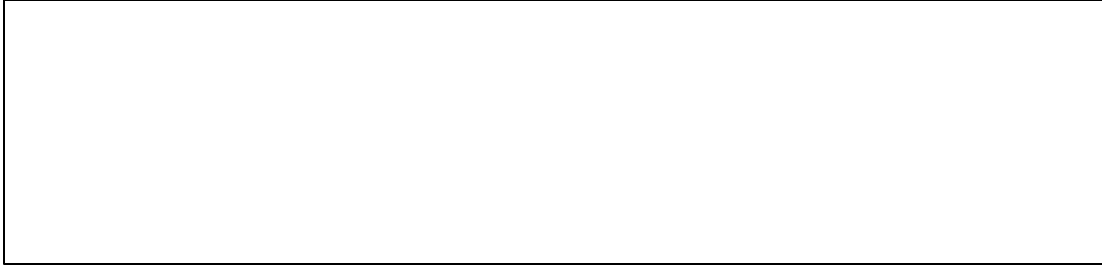
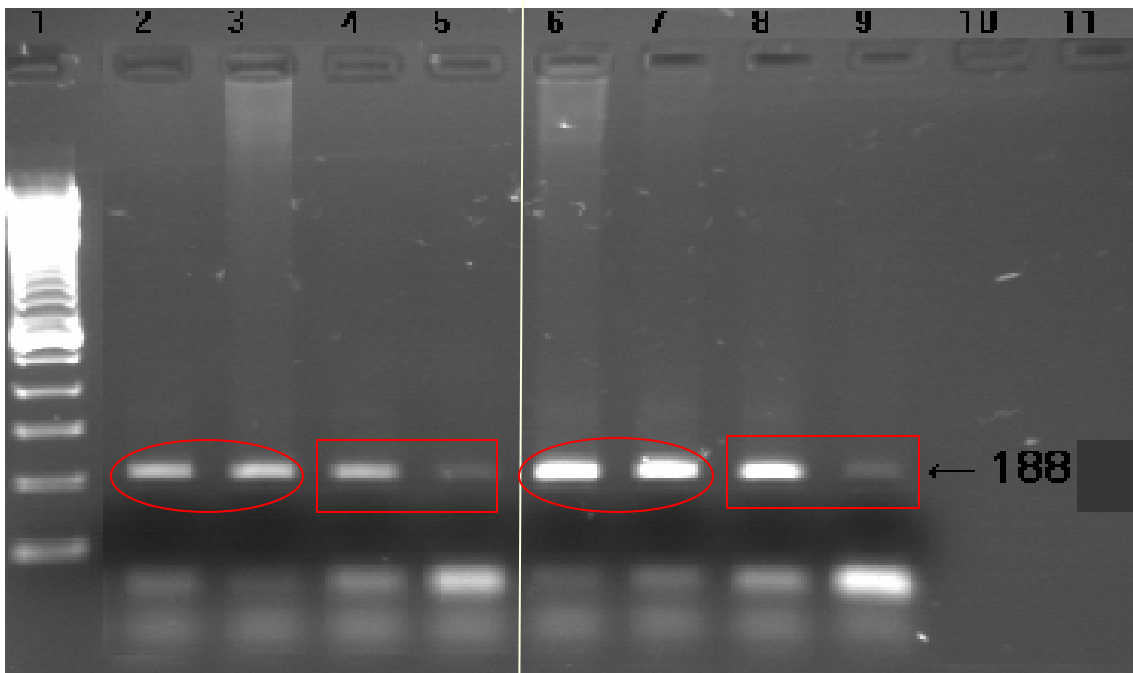


Figure 1. DNA extraction from experimentally infected mosquitoes: Comparison of boiling and phenol-chloroform methods



Size of mosquito pool

The amount of mosquitoes in each pool was varied (see Figure 2) and the mosquitoes were individually examined. These experiments were repeated and it was decided to work with more than five mosquitoes per pool.

Laboratory conditions

In an attempt to standardize laboratory conditions regarding parasitic sensitivity for mf detection, experiments were conducted to determine the optimum primer amounts (concentration of mf DNA), PCR program (number of amplification cycles), and concentration of reagent (Taq [Thermus aquaticus] / dNTPs [Deoxynucleotide-triphosphates], buffer) (see Figure 3).

Figure 2. DNA extraction from non-infected mosquitoes + mf

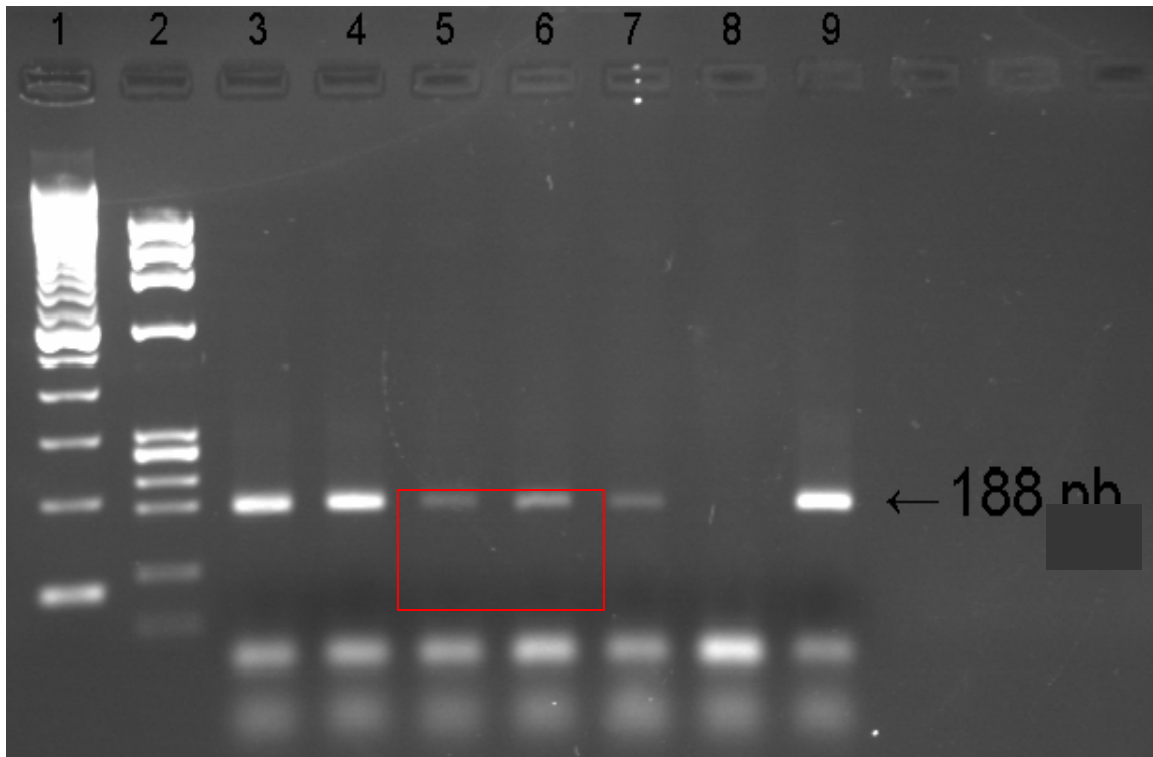


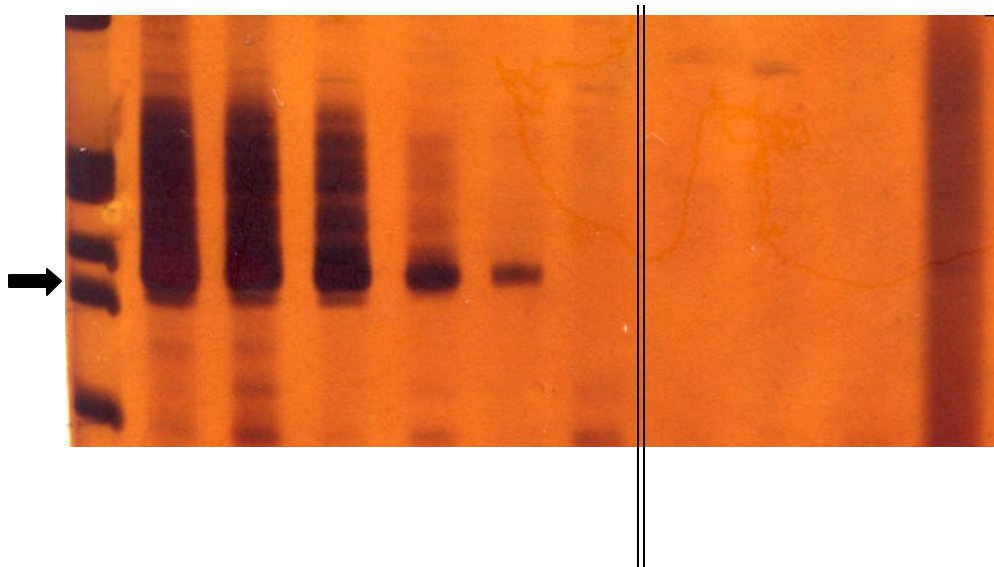
Figure 3. Standardization of primer (mf DNA) amount: Concentration varied to select optimum amount



Detection of mf

- Specificity and sensitivity of PCR
 - Amount of parasites
 - 100 mf
 - 50 mf
 - 10 mf
 - 5 mf
 - 1 mf
 - DNA concentration
 - 12 ng
 - 1200 pg
 - 120 pg
 - 12 pg
 - 1.2 pg

Figure 3. Specificity and sensitivity of reaction in detection of *W. bancrofti* and *D. immitis* DNA by PCR



Conclusions

The PCR standardization process was time-consuming but brought important results regarding the technique eventually selected for laboratory extraction: the boiling method (100°C/10 min). The boiling method was chosen for PCR-based assays for monitoring *W. bancrofti* infection to determine natural infection in mosquito vectors because it is quick, simple, low-cost, non-toxic, has good sensitivity, and is reproducible. In terms of specificity, however, *D. immitis* larvae in infected mosquitoes of the same species are not revealed.

PHASE II

Process

Belém

Research steps in Belém included

- training
 - insect collection (xenomonitoring²)
- screening and recording data for collected mosquitoes (species, amount, number of females)
- mosquito storage (5 mosquitoes/tube)
- mosquito freezing (–23°C)
- mosquito delivery: Maceió

Maceió

- Mosquito registration
- DNA extraction
- PCR testing
- Analysis of results

After training was conducted in xenomonitoring, mosquitoes were collected using this technique and taken to the laboratory, where the females were separated from the males. The mosquitoes were collected in the same places where active case identification was done, in districts and localities in the City of Belém traditionally endemic for lymphatic filariasis (LF). Once a reasonable amount of mosquitoes had been collected, the sample was divided up (according to the specific examination that was to be done using each different population sample), stored (5 mosquitoes/tube), and frozen (–23°C). Cq mosquitoes were also examined, counted, and sent to Maceió, where they were processed by PCR (see Figure 4).

In Maceió, all the material was collected and codified according to a set protocol (including data on where and when the collection took place). The mosquitoes were then registered, their DNA was extracted and tested with PCR, and the results were analyzed. The results of each sample with regard to vector infection showed that the human infection results were independent of the results from the mosquito pools, which were consistently negative: No infected mosquitoes were found.

Samples

The samples examined included (1) mosquitoes x thick blood smear, (2) xenomonitoring x thick blood smear, and (3) xenomonitoring x immunochromatographic test (ICT). The collection for the first sample took place from 2002–2003; almost 900,000 thick blood smears were tested (see Table 1). Results were compared with the results from the survey of human infection using the same thick blood smear test.

² Chadee, D.D.; Williams, S.A.; Ottesen, E.A. *Annals of Tropical Medicine and Parasitology*, 96 (Suppl. 2), p. S47–S53, 2002.

Figure 4. Extracted DNA of mosquitoes from Belém

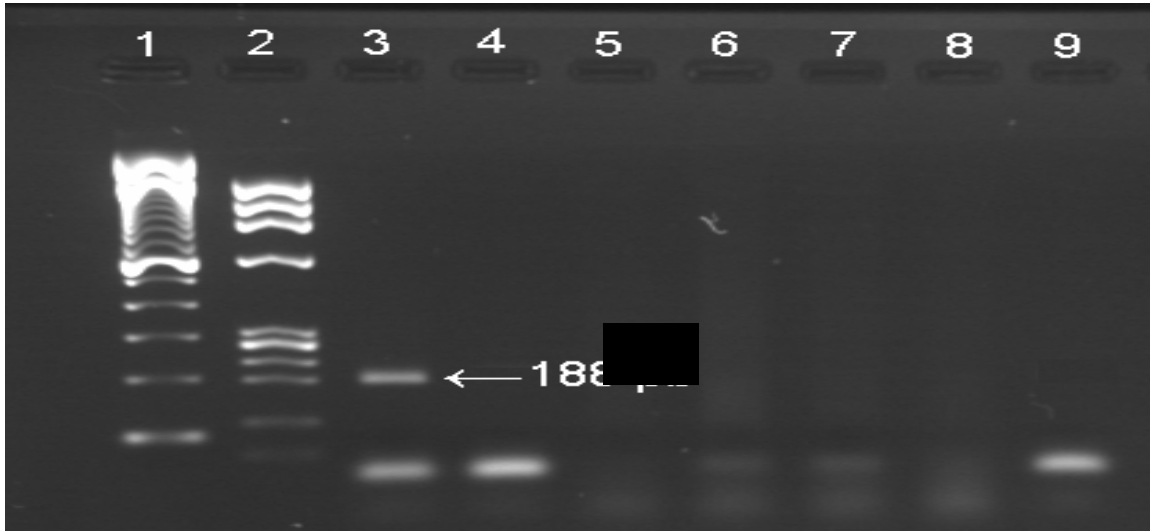


Table 1. Mosquitoes x thick blood smear (2002–2003)*

District	Site	N° of mosquitoes examined	Positive pools
DAICO	Campina Cruzeiro Ponta Grossa Paracuri	1,250	0
DAMOS	Baía do Sol Carananduba Murubira Vila Maracajá	1,150	0
DAOUT	Fidélis Água Boa Brasília Itaituá	300	0
DABEN	Cabanagem Una Coqueiro Tapanã	2,350	0
DAENT	Guanabara Águas Lindas Marambaia	1,700	0
DASAC	Sacramenta Telégrafo Pedreira	2,350	0
TOTAL		9,100	0

In another sample (see Table 2), 21,900 units were examined by xenomonitoring. None were found to be positive for mf. In the same areas, more than 600,000 thick blood smears were collected, and all were negative.

Table 2. Xenomonitoring x thick blood smear (2003–2004)*

In the last sample, the local mosquitoes collected for xenomonitoring were separated out and adult males were examined using ICT cards (2,816 examinations), as shown in Table 3. In this sample 9,800 mosquitoes were examined and no cultivation of parasites was found.

Conclusions

- The DNA extracted from collected mosquitoes showed no PCR positive reaction to *W. bancrofti* (i.e., there were no parasite larvae in the mosquitoes).
- The negative results regarding the incidence of natural infection suggest the interruption of *W. bancrofti* transmission in the area.

Validation work, which has consistently revealed negative results, is ongoing. Similar work is ongoing in Maceió and Salvador.

