A UNIVERSALLY APPLICABLE INTERNAL STANDARD FOR PCR DETECTION OF *WUCHERERIA BANCROFTI* IN BIOLOGICAL SAMPLES

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Summary :

A PCR-based assay have been previously described to detect *Wuchereria bancrofti* in mosquitoes and in human blood samples. However, the efficiency of PCR amplification may vary between samples depending on the presence of PCR inhibitors, leading sometimes to false negative results. To overcome this drawback, an internal standard plasmid (pWB11) was constructed. It can be added to each PCR reaction for coamplification along with the target *W. bancrofti* DNA (*Ssp*I DNA repeat) using the same pair of primers. PCR products from *W. bancrofti* DNA or from pWB11 are 34 bp different in size and can be visualized either on agarose gel or by DNA ELISA using two different oligonucleotides probes.

KEY WORDS : *Wuchereria bancrofti*, PCR, internal standard, DNA-ELISA.

Résumé : UN STANDARD INTERNE UNIVERSEL POUR LA DÉTECTION PAR PCR DE *WUCHERERIA BANCROFTI* DANS LES ÉCHANTILLONS BIOLOGIQUES

Un test d'amplification génique (PCR) pour détecter Wuchereria bancrofti *dans les moustiques et le sang humain a été précédemment décrit. L'efficacité de la réaction de PCR peut cependant varier d'un échantillon à l'autre, en raison de la présence d'inhibiteurs de PCR, entraînant parfois des faux-négatifs. Un plasmide (pWB11), qui sert de standard interne de PCR, a été construit par clonage de la séquence d'ADN répété cible de* W. bancrofti *et insertion de 34 pb par mutagénèse. La séquence clonée dans le plasmide peut être coamplifiée avec la séquence cible de* W. bancrofti, *en utilisant les mêmes amorces. Les deux produits PCR peuvent être détectés soit par électrophorèse en gel d'agarose, soit par ADN-ELISA en utilisant deux sondes nucléotidiques spécifiques de chaque produit.*

MOTS CLÉS : Wuchereria bancrofti, *PCR, standard interne, ADN-ELISA.*

INTRODUCTION

The world burden of human lymphatic filariasis is estimated to be at least 120 million people infected mainly by *Wuchereria bancrofti* and to a lesser extent by *Brugia malayi* or *Brugia timori* (Ottesen & Ramachandran, 1995). Evaluation of control by chemotherapy campaigns is based on detection of the parasite in humans and on monitoring of the parasitological infection of mosquito populations. Development of molecular techniques for detection of filarial parasites in pools of vectors is an objective of the World Health Organization (Ottesen & Ramachandran, 1995), to avoid fastidious and cumbersome dissection and microscopic examination of thousands of mosquitoes. In addition, PCR detection of the filarial parasites in blood with species specific probes might be of help in areas where several filarial species coexist.

PCR-based assays have been developed for detecting *Wuchereria bancrofti* in mosquitoes (Chanteau *et al.*, 1994; Nicolas *et al.*, 1996) and in human blood samples (Williams *et al.*, 1996; McCarthy *et al.*, 1996). The target sequence (*Ssp*I DNA repeat), identified from *W. bancrofti* genomic DNA (Zhong *et al.*, 1996), is specific of the *Wuchereria* genus and ubiquitous to all geographical isolates of *W. bancrofti* tested. A DNA-ELISA version of the PCR assay has also been developed (Nutman *et al.*, 1994), based on the use of biotynilated primers, enabling the quantification of *W. bancrofti* DNA in blood samples (Williams *et al.*, 1996).

The efficiency of PCR amplification may vary from sample to sample, depending on the quality of DNA preparations. Some PCR inhibitors from mosquitoes or human fluids may not be removed completely leading to false negative results. We have therefore constructed an internal standard which can be added to each PCR reaction for coamplification along with the target *W. bancrofti* DNA using the same pair of primers. Quality control can be determined either by visualization of PCR products on agarose gel or by DNA-ELISA using two oligonucleotide probes.

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MATERIALS AND METHODS

Construction of the internal standard plasmid

Genomic DNA was prepared from *W. bancrofti* var. *pacifica* microfilariae (Mf) (Zhong *et al.*, 1996), and the *Ssp*I DNA repeat was amplified by PCR using primers designated NV1 and NV2 (Nicolas *et al.*, 1996; Zhong *et al.*, 1996). Additionnally a 19 mer species-specific probe (P1) that hybridizes only to the *Ssp*I DNA repeat of *W. bancrofti*, was synthetized (Williams *et al.*, 1996). The *Ssp*I PCR product was made blunt-end with Klenow fragment then 5'-phosphorylated with T4 polynucleotide kinase and ligated into the dephosphorylated plasmid pTZ18 between *Hin*cII and *Ecl*136II restriction sites with T4 DNA ligase. Recombinant plasmid was obtained in *Escherichia coli* TG2 cells and is designated pWB01. The *W. bancrofti* insert of plasmid pWB01 was then mutagenised by inserting a 34 bp sequence within the hybridization site of probe P1. The inserted sequence was choosen in the polylinker sequence deleted from pTZ18. Mutagenesis was performed using Muta-Gene™ phagemid *in vitro* mutagenesis kit (Biorad, Ivry s/Seine, France) and a 55-mer oligonucleotide which sequence was : 5' - TAA GGT TAT ACG GTC GAC TCT AGA GGA TCC CCG GGT ACC GAG CTC CAA GCA AAC A - 3'. The resulting plasmid, pWB11, is the internal standard plasmid (Fig. 1). A 20-mer probe (P2) which hybridizes to the inserted sequence was synthetized. Its sequence is: 5' - TCG GTA CCC GGG GAT CCT CT - 3'. Oligonucleotides were synthetized by Genosys Biotechnologies (Cambridge, England).

DNA sequence of the inserts

Plasmids pWB01 and pWB11 were prepared from *E. coli* TG2 cells using the Plasmid Midi Kit from Qiagen (Chatsworth, CA). The *W. bancrofti* DNA inserts of both plasmids were sequenced by thermal cycle sequencing using the Circumvent Thermal Cycle DNA sequencing kit from New England Biolabs (Beverly, MA) with T3 and T7 primers. The sequencing reactions were conducted with the thermal profile of 25 cycles of 20 sec at 94 °C, 20 sec at 55 °C and 20 sec at 72 °C.

Sensitivity of *W. bancrofti* DNA detection in coamplification assays with internal standard

The sensitivity of PCR assay was assayed separately on serial dilutions of purified pWB01, pWB11 and genomic *W. bancrofti* genomic DNA. Then coamplifications assays were carried out with a range of genomic *W. bancrofti* DNA and fixed concentrations of pWB11 plasmid to check whether competition between target DNA and internal standard occurred.

Coamplification assays on mosquito and human blood extracts

DNA was extracted from pools of 50 unparasitized *Aedes polynesiensis* and pools composed of 49 unparasitized *A. polynesiensis* and a single *A. polynesiensis* carrying 1 to 2 Mf (Nicolas *et al.*, 1996). DNA was also extracted (Williams *et al.*, 1996) from 100 µl blood samples collected from either unparasitized or from microfilaremic Polynesian individuals. PCR was carried out on extracts without or in presence of 0.5 or 2 fg of pWB11 plasmid.

PCR conditions

PCR reactions were carried out in a final volume of 50 µl and included two units of Taq polymerase (Promega), 400 pM of both primers NV1 and NV2 and 200 µM of each dideoxynucleotide in 50 mM KCl, 10 mM Tris-HCl (pH9), 0.1 % Triton X100 and 1.5 mM MgCl2. The temperature programme for the PCR was 5 min at 92 °C, then 35 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. The volumes of templates added were 1 µl for internal standard, 1 µl of *W. bancrofti* DNA, 5 µl mosquito extract or 2 µl blood extract. Primer NV2 was 5' end-labelled with biotin to allow detection of the PCR products either by ethidium bromide staining on a 1.5 % agarose gel, on which 10 µl of PCR reaction were loaded, or by DNA-ELISA.

DNA-ELISA

The two biotin-labelled PCR products, 191 bp and 225 bp in length, were then quantified on microtitre plates coated with streptavidin. Product from each PCR reaction was added to four separate wells, so that the PCR products could be hybridized in duplicate by either probe P1 or P2 for specific detection of the 191 bp or 225 bp, respectively. Labelling of probes and quantification were carried out as described in (Williams *et al.*, 1996). Preliminary experiments showed that hybridization should be performed at 55°C to avoid cross-reactivity between probe P1 and the PCR product from internal standard. Blank was obtained with wells containing all reagents except PCR products. Results were expressed as optical density (492 nm) substracted of blank values.

RESULTS

Characterization of the internal standard plasmid and PCR products

The maps of plasmids pWB01 and pWB11 are illustrated in Figure 1A. PCR with NV1 and NV2 primers produces a 191 bp DNA fragment from pWB01, which represents one of the 500 copies pre-

sent on *W. bancrofti* genomic DNA. PCR on pWB11 yields a 225 bp fragment, the sequence of which is given in Figure 1B.

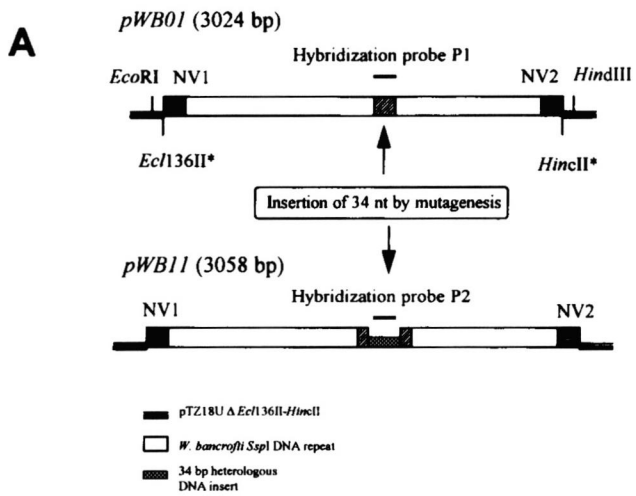
Sensitivity of *W. bancrofti* DNA detection in coamplification assays with pWB11

Results are illustrated in Figure 2A. The limit of detection by PCR of pWB01 and pWB11 when amplified separately was 0.5 fg for both plasmids (data not shown) on agarose gel, while that of *W. bancrofti* genomic DNA was 5 fg on agarose gel (Panel 1) or by DNA-ELISA (Panel 3). Coamplification of dilutions of *W. bancrofti* DNA with 0.5 fg of pWB11 were then carried out. The sensitivity of detection of *W. bancrofti* DNA PCR product (191 bp), either by agarose gel staining (Panel 2) or by DNA-ELISA with probe P1 (Panel 4) was not significantly modified by the presence of the internal standard in the reaction. In contrast, the detection of the PCR product from the internal standard (225 bp) was lowered when *W. bancrofti* DNA concentration increased in the reaction mix (Panels 2 and 4).

Coamplification assays on mosquito and blood extracts

Results are illustrated in Figure 2B. The sensitivity of detection of the pWB11 can vary between mosquito extracts (Left panel). In one extract (lanes *a, b, c*) a faint 225 bp band was observed with 2 fg of plasmid as template while a stronger was observed with only 0.5 fg as template in the second extract (lanes *d, e, f*). Lanes g to l illustrated the PCR products obtained from three pools of 50 mosquitoes including a single *W. bancrofti* infected mosquito. They were run by PCR in absence (lanes *g, i, k*) or in presence of 0.5 fg internal standard (lanes *h, j, l*). No band was obtained with extract 1 (lanes *g, h*) even when standard plasmid was added, which shows that the PCR reaction was inhibited. Extract 2 (lanes *i, j*) was positive for both *W. bancrofti* and the standard. In contrast, the extract 3 (lanes *k, l*) which did not yield a 195 bp product, even in absence of standard (lane *k*) while the standard was strongly amplified (lane *l*), can be considered as negative for the presence of *W. bancrofti* DNA.

Results with blood extracts are shown in Figure 2B (Right panel). Lanes *a, b, c* show an extract from



B

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CGTGATGGCA TCAAAGTAGC GTAAGGGAAT TGTTTTTTTA ATATTTTCAA  50
NV1
GTATGAATGG AATTTTTAGC AATTTTTTTG TTTATATTTT TATTTGAATT 100

ATTTTTTTTT TTTTTGTTTG CTTGGAGCTC GGTACCCGGG GATCCTCTAG 150
                                 Hybridization probe P2
AGTCGACCGT ATAACCTTAT TTTTTAATCT TTTTTAATTT TTTTAGTTTT 200

TTTGTTGTCT TATGGTAAGT GAGGG
                          NV2
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Fig. 1. — Characterization of the internal standard plasmid pWB11.

A. Maps of plasmid pWB01 and of the internal standard plasmid pWB11 obtained by mutagenesis from pWB01. Shaded boxes indicate hybridization site of probe P1 inside the cloned *W. bancrofti* DNA fragment. Asterix (*) indicate the restriction sites lost by cloning *W. bancrofti* DNA fragment.

B. Nucleotide sequence of the target *W. bancrofti* DNA in plasmid pWB11. The sequence is 225 basepairs in length. The underlined sequences at the ends of the sequences are the primers NV1 and NV2 used for polymerase chain reaction amplification. Nucleotides in standard letters correspond also to the sequence of the target *W. bancrofti* in plasmid pWB01. Nucleotides in italics were inserted by mutagenesis into pWB01 plasmid and included an internal hybridization probe site designated as P2 and specific of pWB11.

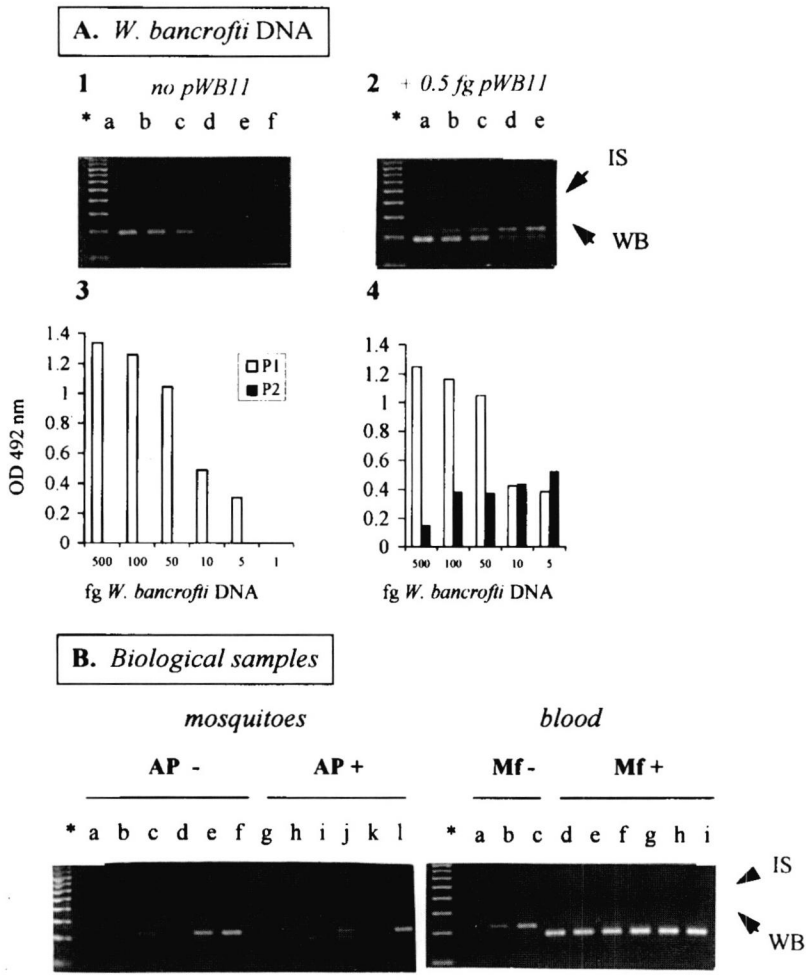

Fig. 2. — Coamplifications assays of internal standard plasmid pWB11 with *W. bancrofti* genomic DNA (A) or DNA extract from biological samples (B).

A. Amplification of the *Ssp*I DNA repeat from *W. bancrofti* genomic DNA either alone (Panels 1 and 3) or in presence of 0.5 fg of the internal standard plasmid pWB11 in coamplification reactions using primers NV1 and NV2 (Panels 2 and 4). PCR products were detected either by agarose gel staining (Panels 1 and 2) or by DNA-ELISA (Panels 3 and 4) using two oligonucleotide probes P1 and P2 specific respectively of genomic DNA or internal standard product. * : molecular weight marker; WB: product from *W. bancrofti* genomic DNA; IS: 225 bp product from pWB11.

B. Application of internal standard plasmid pWB11 in detection of *W. bancrofti* in *A. polynesiensis* mosquitoes (Left) or human blood samples (Right). PCR products are visualized on agarose gel.

Mosquito DNA extracts : AP–: 2 pools (*a-c, d-f*) of parasite-free *A. polynesiensis* run in PCR without (lanes *a, d*) or with 0.5 fg (lanes *b, e*) or 2 fg (lanes *c, f*) of pWB11. AP+ : 3 pools (*g-h, i-j, k-l*) containing a single infected mosquito, run in PCR without (lanes *g, i, k*) or with 0.5 fg pWB11 (lanes *h, j, l*).

Blood extracts : Mf– : extract from uninfected blood run with 0 (lane *a*), 0.5 fg (lane *b*) or 2 fg pWB11 (lane *c*). Mf+ : extracts from blood containing 5 Mf/ml (lanes *d-f*) and 48 Mf/ml (lanes *g-i*). Extracts were run with 0 (lanes *d, g*), 0.5 fg (lanes *e, h*) or 2 fg pWB11 (lanes *f, i*). * : molecular weight marker; WB: product from *W. bancrofti* DNA; IS : 225 bp product from internal standard.

unparasitized blood sample which was well prepared since as few as 0.5 fg of standard could be detected. The two other extracts (lanes *d, e, f* and lanes *g, h, i*) were from blood samples containing 5 and 48 Mf/ml respectively. It shows that the amplification of the internal standard (even at 2 fg) was inhibited by a high amount of *W. bancrofti* Mf DNA.

For mosquito and blood samples, detection by DNA-ELISA raised similar results than agarose gel detection (data not shown).

DISCUSSION

Detection of *W. bancrofti* larvae in mosquitoes by PCR poolscreening is a powerful tool for monitoring infection rate of mosquito populations or for detecting Mf in blood samples (Nicolas *et al.*, 1996; Williams *et al.*, 1996; Zhong *et al.*, 1996). However, DNA preparations from biological samples need to be performed carefully to avoid the presence of any PCR inhibitor and results may differ depending on the quality of DNA extraction. It is therefore necessary to check the absence of PCR inhibitors in each DNA sample to avoid false negatives. The strategy used in this study allows to amplify the internal standard and *W. bancrofti* target DNA with the same pair of primers, and to detect the two PCR products either on agarose gel, as their size is different, or by DNA-ELISA using two specific probes.

The sequence of the *W. bancrofti Ssp*I DNA repeat cloned in plasmid pWB01 differs slightly from the sequence published (Zhong *et al.*, 1996) and probably is an other copy. Since the internal standard is amplified with NV1-NV2 primers, it can be used worldwide and might allow standardization of data between *W. bancrofti* endemic areas. Moreover, it can be produced easily with conventional techniques of plasmid preparation.

Amplification of internal standard in several extracts of mosquitoes (Fig. 2B) demonstrated that there is some variations in the quality of the extracts regarding the presence of inhibitors. If internal standard is not amplified in an extract, a lower volume of template could

be added to the reaction or if necessary, the DNA could be reextracted.

The sensitivity of the PCR assay to detect genomic DNA is ca. ten times higher (50 fg) than that of detection of plasmids pWB01 and pWB11 (0.5 fg). However, the number of copies per kb is very close on the plasmids and on genomic DNA (0.3 copy/3 kb). This shows that target sequence might be less accessible to primers on genomic DNA than on plasmids.

The amount of DNA per microfilaria was estimated to be ~ 100 pg (Zhong *et al.*, 1996). In a pool of mosquitoes containing a single Mf, the amount of DNA added to the PCR reaction should be 1 to 5 pg depending of the volume of extract (1 to 5 µl) added to the reaction, if parasite DNA was completely extracted from mosquitoes. Therefore the detection limit of the assay (50 fg of *W. bancrofti* DNA) is beyond the necessary limit. However, the success of DNA extraction from mosquitoes needs also to be checked by including in the series of extraction a positive control, which could be a pool of unparasitized mosquito containing a low number of parasitized mosquitoes. The amount of standard plasmid used should be minimal to avoid competition between standard and *W. bancrofti* DNA putatively present. To eliminate this risk, we recommend to run two tubes per extract. One tube should contains only the sample to analyze and should be free of standard and the other should contain the sample and the standard.

Basically, DNA-ELISA gives similar results than agarose gel staining but does not seem to be more sensitive. One of its main advantage is the quantification of DNA (Zhong *et al.*, 1996). For epidemiological assessment, agarose gel staining might be simpler and less expensive to implement than DNA ELISA in endemic countries. Finally, the strategy used to construct internal standard plasmid for PCR can be used for other parasites.

ACKNOWLEDGEMENTS

We thank G. Milon (Institut Pasteur) for critical review of the manuscript and S.A. Williams (Smith College, Northampton, MA) for helpful discussion. Financial support was provided by Institut Pasteur which is a pleasure to acknowledge.

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Reçu le 22 mars 1997
Accepté le 9 juin 1997