

An alternative strategy to generate coding sequence of macrophage migration inhibitory factor-2 of *Wuchereria bancrofti*

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Background & objectives: Different developmental stages of *Wuchereria bancrofti*, the major causal organism of lymphatic filariasis (LF), are difficult to obtain. Beside this limitation, to obtain complete coding sequence (CDS) of a gene one has to isolate mRNA and perform subsequent cDNA synthesis which is laborious and not successful at times. In this study, an alternative strategy employing polymerase chain reaction (PCR) was optimized and validated, to generate CDS of Macrophage migration Inhibitory Factor-2 (*wbMIF-2*), a gene expressed in the transition stage between L3 to L4.

Methods: The genomic DNA of *W. bancrofti* microfilariae was extracted and used to amplify the full length *wbMIF-2* gene (4.275 kb). This amplified product was used as a template for amplifying the exons separately, using the overlapping primers, which were then assembled through another round of PCR.

Results: A simple strategy was developed based on PCR, which is used routinely in molecular biology laboratories. The amplified CDS of 363 bp of *wbMIF-2* generated using genomic DNA splicing technique was devoid of any intronic sequence.

Interpretation & conclusions: The cDNA of *wbMIF-2* gene was successfully amplified from genomic DNA of microfilarial stage of *W. bancrofti* thus circumventing the use of inaccessible L3-L4 transitional stage of this parasite. This strategy is useful for generating CDS of genes from parasites that have restricted availability.

Key words Coding sequence - exon assembly - filarial genome database - macrophage migration inhibitory factor - polymerase chain reaction - *Wuchereria bancrofti*

Human lymphatic filariasis (LF) is a parasitic disease caused mainly by lymphatic filarial parasite *Wuchereria bancrofti* and partly by *Brugia malayi* and *B. timori*¹. The disease is prevalent mainly in tropical and sub-tropical countries. The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was launched in 2000 and after completing 13 years has resulted in substantial reduction in LF burden².

Research on lymphatic filariasis is mainly based on a zoonotic parasite, *Brugia malayi* as a model organism. The reason is the availability of an animal model *Mastomys coucha* to carry *B. malayi* infection and its developmental stages in laboratory³. This model provides a continuous source of DNA or proteins to study various molecular aspects of lymphatic filariasis. But, the source of developmental stages of

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W. bancrofti, a major causal organism of lymphatic filariasis, is not available for such studies. Beside this limitation, isolation of mRNA and generation of cDNA from certain stages of *W. bancrofti* is not possible due to difficulty in obtaining them⁴. Microfilariae of *W. bancrofti* can be obtained from carriers, but later stages (L1, L2 and L3) need to be raised in vectors which is not possible in all the laboratories, while other human stages are not accessible. Hence, obtaining mRNA from these stages to generate complete coding sequence (CDS) through reverse transcription (RT)-PCR is difficult. Further, conventional method of generating CDS requires mRNA isolation and RT-PCR kits, which are expensive, apart from the method being laborious and less successful at times. And, for successful amplification of a particular cDNA, high level of expression of the gene is often required. The presence of RNases in the tissue samples decreases the success rate of cDNA amplification by RT-PCR. Genomic DNA splicing technique of generating cDNA directly from genomic DNA offers to circumvent these problems^{5,6}. The data from recently ongoing genome sequencing project of *W. bancrofti* supported by the National Institute of Allergy and Infectious Disease, National Institutes of Health funded Genome Sequencing Centre for Infectious Diseases at the Broad Institute (<http://www.broadinstitute.org>) offer an opportunity to generate cDNA of different genes directly from genomic DNA. In the present study, the cDNA of Macrophage migration Inhibitory Factor-2 (*wbMIF-2*), a gene expressed in the inaccessible molting stage (from L3 to L4) of *W. bancrofti*⁷, was amplified using genomic DNA of microfilarial stage. The gene was identified from Filarial Genome Project sequence database (<http://www.broadinstitute.org/>) and its CDS was amplified directly from genomic DNA by using a simple genomic exon splicing strategy. Amplified coding sequence of *wbMIF-2* can be further used for cloning, expression and functional characterization.

Material & Methods

The study was conducted in the Division of Microbiology and Immunology of Vector Control Research Centre (ICMR), Puducherry, India.

Gene scanning and annotation: Genome sequence of *W. bancrofti* was obtained from the Filarial Worms Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology (MIT), USA (<http://www.broadinstitute.org/>). The gene was identified and annotated using MEGA 5 software⁸

by alignment of the cDNA sequence of Macrophage migration Inhibitory Factor-2 of *B. malayi* (BmMIF-2, AY004865.1), obtained from National Centre for Biotechnology Information (NCBI) databank (<http://www.ncbi.nlm.nih.gov/>).

Designing of primers: Four primers were designed for the amplification of *wbMIF-2* using Gene runner (<http://www.generunner.net/>) by optimizing melting temperature (T_m) and loop formation parameters. One set of primers (MIF-2F1 & MIF-2R1) was designed for the amplification of full length of *wbMIF-2* gene and another set of overlapping primers (MIF-2OF1 & MIF-2OR1) designed on the basis of exonic sequences. The primer sequences are: MIF-2F1: 5'- ATG CCG CTG ATA ACG CTT G- 3'; MIF-2R1: 5'- TTA TTT CTT CAT AAG CTC TTT CAT TGT - 3'; MIF-2OF1: 5'- TTA CAG TGG TTA AAT CGA TTG GAT C-3'; MIF-2OR1: 5'-CAC TGT AAG ACA TGA TGG ATT TTC A-3'.

PCR amplification of full gene and exons: The full length *wbMIF-2* gene of 4.275 kb was amplified using genomic DNA extracted from microfilariae (n=50) of *W. bancrofti*, purified from blood⁹. The microfilaraemic blood (0.5 ml) collected at night from a carrier residing in Puducherry was used for DNA extraction. The DNA extraction was carried out using QIamp DNA micro kit (Qiagen, Germany) and the concentration was estimated in GenQuant (Amersham Bioscience, USA). The 50 µl of PCR reaction mix contained 1.5 µl of 10pmol of each primer (MIF-2F1 and MIF-2R1), 2x of PrimeSTAR Max master mix (TAKARA, Japan) and 50 ng of genomic DNA template. The PCR cycle parameters were as follows: initial denaturation at 98°C for four min followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 50°C for 30 sec, extension at 72°C for two min with final extension at 72°C for 10 min. The amplified full length *wbMIF-2* PCR product was run on 0.8 per cent agarose gel with Ethidium bromide and observed under UV light. The *wbMIF-2* band of 4.275 kb was cut and purified by using a nucleic acid gel purification kit (Macherey-Nagel, Germany). This purified product was used as a template for amplification of the two exons, in separate reactions. The overlapping primers were designed within similar range of T_m to amplify both the exons using the same PCR cycle simultaneously. PCR parameters for amplification of both the exons were as follows: initial denaturation at 95°C for five min followed by 30 cycles of denaturation at 95°C

for one min, annealing at 50°C for 20 sec, extension at 72°C for 30 sec, with final extension at 72°C for 10 min. The 50 µl of PCR reaction for amplification of the two exons contained 1 µl of 10 pmol of primers (MIF-2F1 and MIF-2OR1) and (MIF-2OF1 and MIF-2R1), 0.5 unit of PR polymerase (Merck, India), 20 mmol dNTPs (FinZyme, Finland) and 50 ng purified DNA template. The amplified exons were purified using gel extraction kit and used in the assembly PCR as templates for amplification of the CDS.

Assembly of exons in single PCR reaction: Both the amplified exons were joined together in assembly PCR. The PCR reaction mixture contained 1 µl of 10 pmol of each primer (F1 and R1), 0.5 units of PR polymerase (Merck, India), 20 mmol dNTPs (FinZyme, Finland) and 50 ng of both purified exons as a template. PCR cycle parameters were as follows: initial denaturation at 95°C for five min followed by 35 cycles of denaturation at 95°C for one min, annealing at 50°C for 20 sec and extension at 72°C for 30 sec, with final extension at 72°C for 10 min. The amplified full length of *wbMIF-2* coding sequence was run on 1.5 per cent of agarose gel with ethidium bromide and visualized under UV.

Sequencing confirmation of amplified CDS: The identity of full length *wbMIF-2* coding sequence generated was confirmed by sequencing in ABI 3130Xl sequencer (Applied Biosystem, USA). The sequencing reaction of 10 µl contained 4 µl of Big Dye terminator (Applied biosystem, USA), 2 µl of 1 pmol primer (F1) and 4 µl of nuclease free water. Cycle sequencing parameters were followed as per manufacturer instructions.

Results

The genomic exon splicing strategy is shown in the flow chart (Fig. 1). Identification of MIF-2 of *W. bancrofti* was done on the basis of *B. malayi* MIF-2 cDNA sequence available in NCBI databank, as *W. bancrofti* and *B. malayi* are closely related parasites and have very high similarity of gene sequences. The search based on *B. malayi* MIF-2 cDNA sequence in *W. bancrofti* genome sequence database using MEGA5 software, yielded full length *wbMIF-2* gene of 4.275 kb in the contig ADBV01000282.1. Upon annotation of this, it was found that *wbMIF-2* had two exons of 183 and 180 bp, interspersed with a very large intron of 3.912 kb (Fig. 2). Both the exons are separated by standard rule of GT-AG splicing sites present at the intron endings.

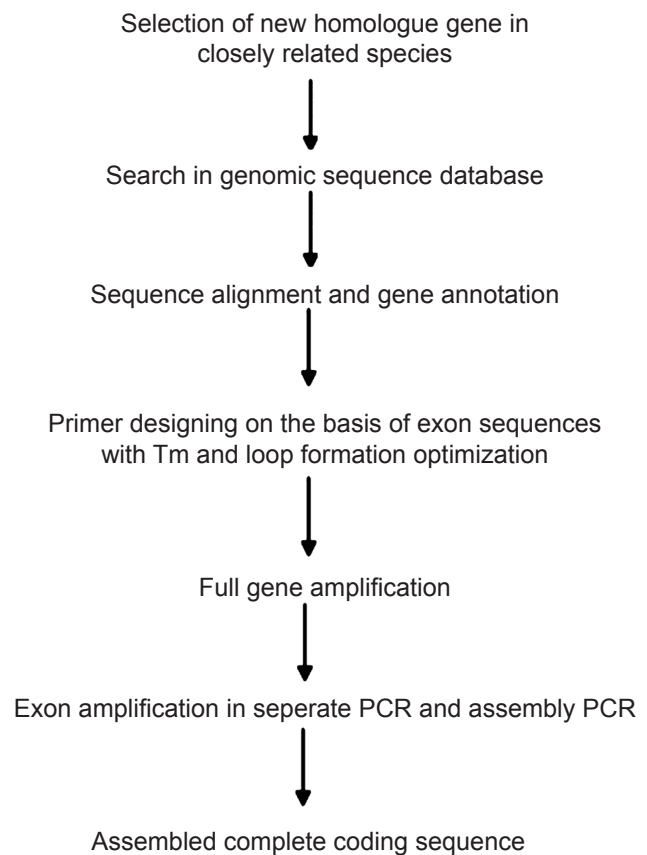


Fig. 1. Flow chart showing the complete process of genomic exon splicing strategy.

The complete nucleotide sequence of *wbMIF-2* from *W. bancrofti* Genome Database was used for primer designing in Gene Runner software with optimization of T_m and loop formation (Fig. 3). The T_m of primers was set in the range of 2°C for each primer. Two sets of primers were designed for the amplification of full length *wbMIF-2* gene and complete *wbMIF-2* coding sequences. In the first PCR full length gene of *wbMIF-2* of 4.275 kb was amplified (Fig. 4a), which was then used as a template for the amplification of the two exons (Fig. 4b) in PCR reaction, instead of using genomic DNA directly, to reduce the need for optimization efforts and enhancing the specificity. The strategy adopted by us required less genomic DNA for exon amplification compared to direct amplification of exons from the genomic DNA. Amplified exons were assembled in a separate PCR reaction using MIF-2F1 and MIF-2R1 primers and both the exons as template, which resulted in 363 bp complete coding sequence (Fig. 4c). The *wbMIF-2* full coding amplicon was sequenced in 3031Xl sequencer and its identity

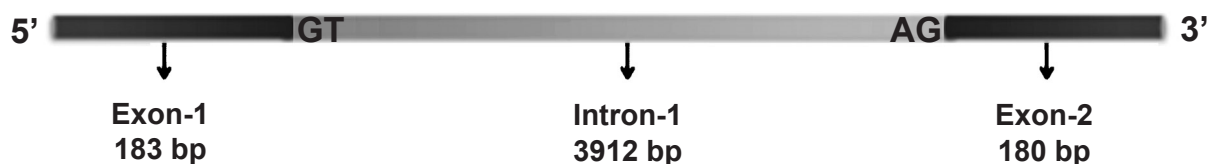


Fig. 2. Organization of *W. bancrofti* MIF-2 gene based on cDNA sequence and *W. bancrofti* contig ADBV01000282.1 from Filarial Genome Project sequences.

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MIF-2F1
ATGCCGCTGA TAACGCTTGC CTCGAACGTT CCCGCGAGTA GATTTCCGAG TGATTTCAAT GTTCAGTTTA CGGAA 75
TACGGCGACT ATTGCGAACG GAGCTTGCAA GGGCGCTCAT CTAAGGCTC ACTAAAGTTA CAAGTCAAAT GCCTT 75

TTAATGGCGA AAATACTAGG AAAACCAACG AGTCGGATAC TTTTACTGGT AATGCCAAAT GCACAACAT CACAT 150
AATTACCGCT TTTATGATCC TTTGGTTGC TCAGCCTATG AAAATGACCA TTACGGTTTA CGTGTGATA GTGTA 150

MIF-2OF1
GGCACAAC TG AAAATCCATC ATGTCTTACA GTGGTTAAAT CGATTGGATC ATTTCCGGCT GATAAAAATA TTGAA 225
CCGTGTGAC TTTTAGGTAG TACAGAATGT CACCAATTTA GCTAACCTAG TAAAAGCCGA CTATTTTTAT AACCT 225

MIF-2OR1
TATTCCTCGT CAATATCAGA ATTCATGAAG AAAACATTGG ATATCGATCC AGCGCATTGT ATCATTCAAT TCTTA 300
ATAAGGAGCA GTTATAGTCT TAAGTACTTC TTTTGTAACC TATAGCTAGG TCGCGTAACA TAGTAAGTAA AGAAT 300

AATTT GGATCCAGAA AATGTTGGATG CAATGGAACA ACAATGAAAG AGCTTATGAA GAAATAA 363
TTAAA CCTAGGTCTT TTACAACCTAC GTTACCTTGT TGTTACTTTC TCGAATACTT CTTTATT 363

MIF-2R1
    
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Fig. 3. Complete coding sequence of Macrophage migration Inhibitory Factor-2 of *W. bancrofti*. Arrows indicate primer sequences designed on the basis of *wbMIF-2* complete coding sequence. Source: *W. bancrofti* genome database (<http://www.broadinstitute.org>).

was confirmed by aligning with *W. bancrofti* genome sequence. The sequence was found to match with a fragment between 12530 nt to 16804 nt, completely in the contig ADBV01000282.1.

Discussion

Parasitic agents remain a major cause of morbidity and mortality in humans in developing countries¹⁰ and lymphatic filarial parasite, *W. bancrofti* is one among them. Basic studies on the molecular aspects of developmental biology need to be continued to support the ongoing global LF elimination programme,

especially to manage the morbidity. But, there are limitations to carry out such studies due to the scarcity of parasite material as certain developmental stages of the parasite are difficult to obtain or inaccessible, and a cyclic animal model is not available and there is no *in vitro* culture system for this parasite. Further, the expression of a particular gene may not be adequate to synthesize high amount of cDNA which limits its amplification in PCR, and isolation of mRNA and cDNA synthesis require much more expertise due to the presence of ubiquitous RNAase and less stability of mRNA¹⁰⁻¹².

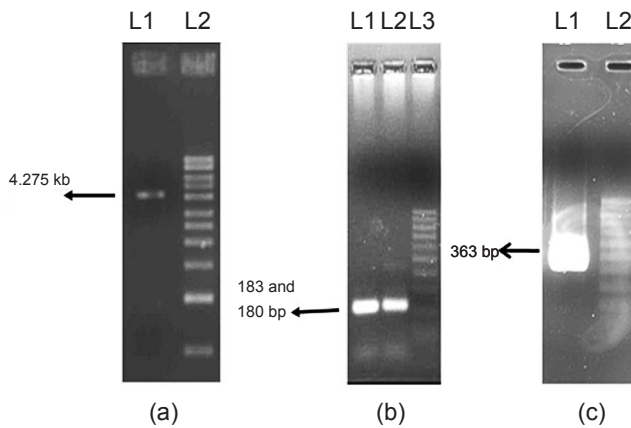


Fig. 4. PCR amplification of (a) *MIF* gene from genomic DNA of *W. bancrofti*. Lane 1, 4.275 kb amplicon, Lane 2- 1 kb marker; (b) *MIF* exons from *MIF* gene amplicon. Lane 1- 183 bp amplicon of first exon, Lane 2- 180 bp of amplicon of second exon, Lane 3- 100 bp ladder; and (c) Assembly of both exons in PCR. Lane 1- 363 bp assembled *wbMIF-2* amplicon, Lane 2- 100 bp ladder.

Genome sequencing of *W. bancrofti* has created an opportunity to understand the mechanism of biological processes such as immune evasion by *W. bancrofti*. In the present study, we amplified the CDS of a gene from *W. bancrofti*, *wbMIF-2* homologue, employing a modified strategy of genomic exon splicing technique which circumvented problem of cDNA synthesis from inaccessible molting L3 stage. In order to achieve this we identified *wbMIF-2* homologue in the *W. bancrofti* genome database. The expression of *wbMIF-2* homologue is specific to L3 molting stage (L3-L4) of *W. bancrofti*¹³ and considered as an immunomodulator at host-parasite interface¹⁴. The process of raising these stages of *W. bancrofti* is difficult, as it requires microfilaraemic blood sample, mosquito rearing facility and *in vitro* culture system. Use of DNA instead of RNA is always better in terms of simplicity, time consumption, reproducing results and cost-effectiveness. PCR based amplification of CDS of *wbMIF-2* by using overlapping primers is easier and cost-effective compared to developmental stage maintenance, mRNA isolation and cDNA synthesis¹⁵. Previously, PCR based exonic assembly strategy has been used for the amplification of complete coding sequence of human genes with several modifications in primer designing and exon amplification¹⁶. However, in this method, human genomic DNA, which can be obtained in adequate quantities, was used directly as template in PCR reaction for amplification of each exon. But in the case of certain developmental stages

of *W. bancrofti* the quantity of DNA available is very limited. To overcome this problem we amplified the full length gene of 4.275 kb and then used it as template for amplification of exons, which in turn were assembled in a separate PCR, to obtain CDS. Use of this strategy for amplification of complete coding sequence of a particular gene of *W. bancrofti* could solve the problem of isolation of mRNA with subsequent cDNA synthesis and need of high quantity of DNA from inaccessible parasitic stages. Further, this method will increase the specificity and reduce the optimization efforts in the amplification of complete coding sequence.

In conclusion, the strategy of genomic exon splicing is a simple, quick, cost-effective and a routinely used PCR based technique to amplify CDS of a particular gene from genomic DNA. Amplified coding sequence can be used for functional characterization of parasite genes and protein coded by such genes. In case of *W. bancrofti*, where certain developmental stages are not available for various studies, it can be a useful approach for generating CDS of various genes.

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Conflicts of Interest: None.

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