Evidence for *Wolbachia* symbiosis in microfilariae of *Wuchereria bancrofti* from West Bengal, India

PRAJNA GAYEN[1,†], SUDIPTA MAITRA[2,†], SUTAPA DATTA[1] and SANTI P SINHA BABU[1,*]

[1]*Parasitology Laboratory,* [2]*Physiology and Biochemistry Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan 731 235, India*

[†]*These two authors have contributed equally to this paper*

**Corresponding author (Fax, +913463-261176; Email, santi_p@rediffmail.com)*

Wolbachia are symbiotic endobacteria that infect the majority of filarial nematodes, including *Wuchereria bancrofti, Brugia malayi* and *Onchocerca volvulus*. Recent studies have suggested that *Wolbachia* are necessary for the reproduction and survival of filarial nematodes and have highlighted the use of antibiotic therapy such as tetracycline/doxycycline as a novel method of treatment for infections caused by these organisms. Before such therapy is conceived and implemented on a large scale, it is necessary to assess the prevalence of the endosymbiont in *W. bancrofti* from different geographical locations. We present data from molecular and electron microscopic studies to provide evidence for *Wolbachia* symbiosis in *W. bancrofti* microfilariae collected from two districts (Bankura and Birbhum) of West Bengal, India.

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1. Introduction

The majority of filarial nematodes including *Dirofilaria immitis, Litmosoides sigmodontis, Onchocerca volvulus, Brugia malayi* and *Wuchereria bancrofti* (Bandi *et al.* 1999; Taylor *et al.* 1999), belonging to the order Spirurida and family Onchocercidae harbour the obligate intracellular endosymbiotic bacteria *Wolbachia* (Casiraghi *et al.* 2000). The contribution of the endobacteria to the development, viability and fertility of filarial nematodes hints at a symbiotic relationship among them. Bandi *et al.* (2001) reported that the transmission of *Wolbachia* is from the maternal source to microfilariae (mf) and the nearly 100% presence in all developmental stages is remarkable. This paves the way for the use of suitable antirickettsial drugs (e.g. tetracycline, doxycycline, etc.) as alternative chemotherapeutic agents (Andre *et al.* 2002). However, it is essential to assess the prevalence of *Wolbachia* in *W. bancrofti* from different geographical locations. In this context, Hoti *et al.* (2003) have reported the occurrence of *Wolbachia* in mf of *W. bancrofti* collected from various geographical areas in southern India.

We undertook a preliminary study to screen for the presence of *Wolbachia* in mf of *W. bancrofti* collected from two districts of West Bengal, Bankura (23° 14′ N and 87° 07′ E) and Birbhum (24° 35′ N and 88° 1′ 40″E), by electron microscopic study of mf and polymerase chain reaction (PCR) for *Wolbachia*-specific 16S rRNA and *Wolbachia* surface protein (WSP) genes.

2. Materials and methods

2.1 *Sample collection and study area*

The present survey was conducted in two rural areas endemic for filaria. Samples were taken from sixteen

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Abbreviations used: dNTP, deoxyribonucleotide triphosphate; EDTA, ethylene diamine tetra acetate; mf, microfilaria; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; $(NH_4)_2SO_4$, ammonium sulphate; PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid; TEN, tris-ethylene diamine tetra acetate-sodium chloride; WSP, *Wolbachia* surface protein

villages each in the two districts of Bankura and Birbhum, West Bengal to determine the prevalence of *W. bancrofti* in the population surveyed. The protocol for this study was reviewed and approved by the Institutional Animal Ethics Committee (Visva-Bharati University) and the Human Ethical Committee of the Sub Divisional Hospital, Bolpur, West Bengal, India. Before taking blood samples, each volunteer was read a consent form and required to sign it.

2.2 *Estimation of prevalence*

Prevalence is a frequently used epidemiological measure of how commonly *W. bancrofti* occurs in a population. The prevalence is calculated by dividing the number of persons who harbour *W. bancrofti* mf in the peripheral blood at a particular time point by the number of individuals examined. It is often expressed as a percentage, calculated by multiplying the ratio by 100.

A mass night blood survey was conducted from November 2006 to October 2008 to detect mf in the peripheral blood by the finger prick method. The work on filariasis is still being continued. Individuals (both male and female) between the ages of 18 and 55 years, and females who were not pregnant or breastfeeding were eligible for this study. We collected blood samples at random from apparently healthy people irrespective of sex and age. Membrane filtration is known to enhance the estimation of prevalence, but it was considered that the risk and discomfort associated with the venepuncture required for this method made it inappropriate for application to the present mass survey. Prior to the survey, a social worker informed the villagers and explained the purpose of the survey to them. The blood collection team visited the selected villages between 2100 and 2300 h for 82 days. A 20 mm^3 peripheral blood smear was collected for subsequent laboratory assessment. Occurrence of mf was recorded by observing the Giemsa-stained blood smear under a microscope. Five millilitre blood samples (heparinized) were collected from 39 and 44 microfilariaemic individuals residing in the districts of Bankura and Birbhum, respectively. Blood samples were diluted (1:1) with chilled phosphate buffered saline (PBS, 10 mM sodium phosphate buffer, 0.9% saline, pH 7.6) and mf were separated using a 5 μm filter membrane (Millipore, USA). The filtrate was centrifuged at 1350 *g* for 5 min at 4°C to pellet down the mf and stored immediately in 100 μl TEN buffer (100 mM Tris-Cl, 5 mM EDTA, 200 mM NaCl, pH 7.5) at –20°C.

It is desirable to conduct a statistical test to examine the assertion that *Wolbachia* occur in *W. bancrofti* mf in

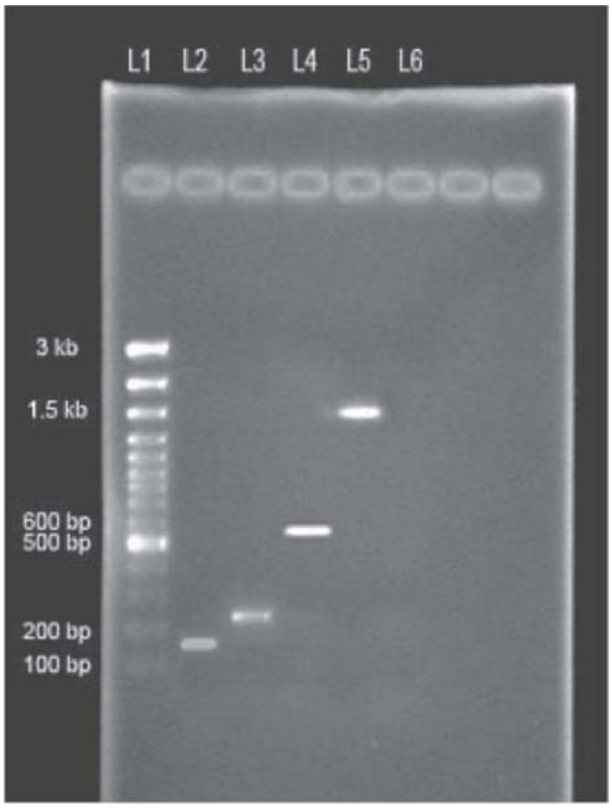

Figure 1. Polymerase chain reaction of genomic DNA of *W. bancrofti* mf using filarial 28S rRNA-specific primers (lane 2), *Wolbachia*-specific 16S rRNA primers (FIL-5 and FIL-6), WSP primers (WSPintF and WSPintR) (lane 3 and lane 4, respectively). Lanes 5 and 6: amplification of *E. coli* DNA using EUBAC-1 and EUBAC-2 as well as FIL-5 and FIL-6 primer pairs, respectively. The migration pattern of the 100 bp DNA ladder is shown at the extreme left (lane 1).

Table 1. Primers used during PCR amplification

BD1A-F	5′- atgaaaggcgttgatatatag-3′	Filaria-specific 28 S rRNA
BD1A-R	5′- gcaagccatgcaagcgttgag-3′	
FIL-5	5′-tgaggaagataatgacgg-3′	*Wolbachia* 16S rRNA-specific
FIL-6	5′-cctctatcctctttcaacc -3′	
WSPintF	5′-tag(ct)tactacattcgcttgca -3′	*Wolbachia* surface protein-specific
WSPintR	5′-ccaa(ct)agtgc(ct)ataaagaac-3′	
EUBAC-1	5′-agagtttgatcctggctcag′-3	Bacterial 16S rRNA-specific
EUBAC-2	5′- aaggaggtgatccagcc ′- 3	

a statistically meaningful number of samples. Therefore, the data were analysed by the *Z*-test using the MS Excel software.

2.3 *DNA extraction and PCR*

Genomic DNA was extracted from mf (approximately 1900 in number) by the phenol/chloroform method (Datta *et al.* 2007). In brief, the mf were digested in 500 μl of lysis buffer (20 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, 100 mM NaCl, 1% β mercaptoethanol [v/v], pH 8.0), proteinase-K (0.1 mg/ml) and incubated in a water bath at 55°C for 3 h. After phenol–chloroform–isoamyl alcohol extraction (25: 24:1) and ethanol precipitation, the final DNA pellet was resuspended in 20 μl of sterile nuclease-free water. PCR was performed using a thermal cycler (Master Cycler Eppendorf, India), in 50 μl of reaction mixture containing PCR buffer (1×) with $(NH_4)_2SO_4$, dNTP mix (2 mM), $MgCl_2$ (2 mM), 1 μM each of forward and reverse primers, 1.5 units of *Taq* polymerase (Fermentas) and template DNA. During thermal cycling, denaturation was done at 95°C for 3 min followed by 35 cycles at 94°C for 45 s, 51°C for 1 min and 72°C for 1 min. Final extension was done at 72°C for 7 min. For amplification of filarial-specific 28 S rRNA, the primer pairs BD1A F and BD1A R (Smith and Rajan 2000) were used. To amplify *Wolbachia* 16 S rRNA, the primers FIL-5 and FIL-6 (Smith and Rajan 2000), and WSPintF and WSPintR (Bazzocchi *et al.* 2000) were used. DNA was also extracted from *Escherichia coli,* a non-rickettsial group of bacteria, and PCR was performed from this bacterial DNA using the primers EUBAC-1 and EUBAC-2 (Smith and Rajan 2000). To ensure the specificity, *Wolbachia*-specific FIL-5 and FIL-6 primers were also used for amplification of non-*Wolbachia* bacterial (*E. coli*) DNA (table 1). PCR products were resolved in 1.2% agarose gel and stained with ethidium bromide to visualize the bands in a gel doc apparatus (Bio Rad).

2.4 *Transmission electron microscopy*

Freshly collected mf (about 200 in number) were fixed with 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) overnight. After rinsing in phosphate buffer, the mf were postfixed with 1% osmium tetraoxide for 2 h, dehydrated in a graded series of ethanol and embedded in spur. Ultrathin sections were examined with a Philips transmission electron microscope (FEI, Technai, Japan). Six samples from each district were processed for detection of *Wolbachia* by transmission electron microscopy (TEM).

3. Results

Prevalence data by district and village are presented in table 2. Prevalence ranged from 0% to 14.5% in district Bankura; the prevalence status in district Birbhum ranged from 4.3% to 15.0% (table 2).

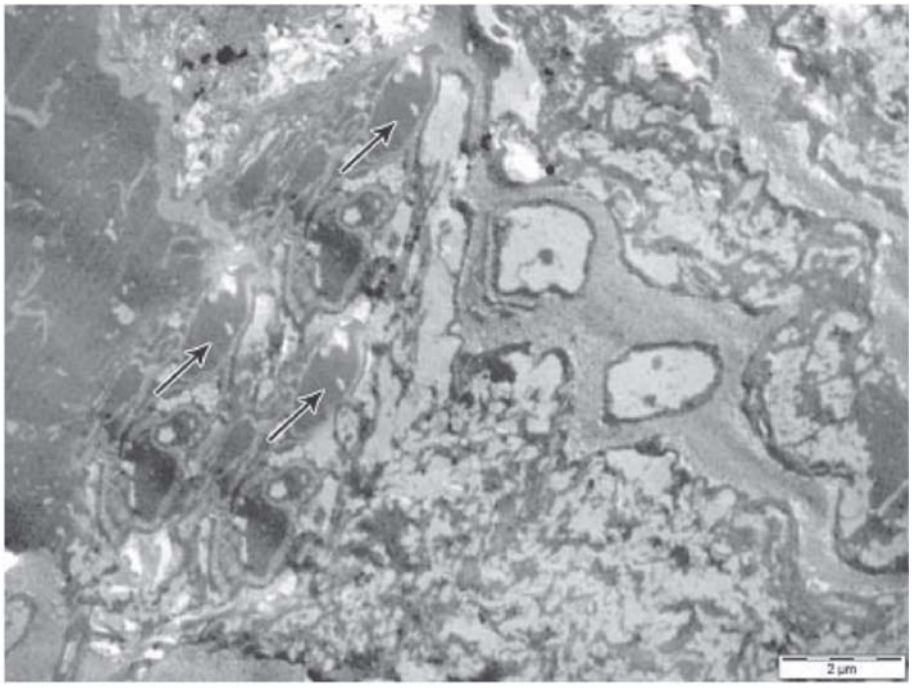

Figure 2. Transmission electron micrograph of *W. bancrofti* microfilaria. The hypodermis shows numerous membrane-bound bacteria (arrows) with ovoid shapes and dense cytoplasm. Scale bar = 2 μm.

Table 2. Microfilarial prevalence data from the population surveyed

District	Villages	Number tested	Number positive	Prevalence (%)	Latitude	Longitude
BANKURA	Moukura	56	6	10.71	23° 35′ N	87° 07′ N
	Baramasia	54	5	9.25		
	Dhararband	67	7	10.44		
	Dhabal	89	9	10.11		
	Dhunara	47	5	10.63		
	Samantamara	29	3	10.34		
	Koima	51	5	9.8		
	Karanjora	96	10	10.41		
	Nabanda	32	4	12.5		
	Katabari	81	10	12.34		
	Kendboni	71	6	8.45		
	Jhantipahari	62	9	14.51		
	Shitpukur	89	11	12.35		
	Aina	10	0	0.00		
	Khorbona	95	8	8.42		
	Ramkanali	16	2	12.5		
Total		945	100			
BIRBHUM	Binuria	113	17	15.04	24° 35′ N	88° 1′ 40″ E
	Bandanga	55	6	10.90		
	Mohisdal	65	9	13.84		
	Majhipara	102	11	10.78		
	Srikantapur	136	16	11.76		
	Muralpur	89	9	10.11		
	Bahiri	54	3	5.55		
	Bangachatra	23	1	4.34		
	Kasba	67	5	7.46		
	Debogram	54	5	9.25		
	Parui	41	6	14.63		
	Panchsowa	36	3	8.33		
	Purandarpur	95	12	12.63		
	Harserandi	84	5	5.95		
	Sangsad	61	8	13.11		
	Sian	55	6	10.90		
Total		1130	122			

In the present study, PCR amplification of *W. bancrofti* genomic DNA with filarial-specific 28 S rRNA primers yielded distinctive bands at 150 bp confirming the quality of template DNA and authenticity of the experimental protocol (figure 1). To confirm the presence of *Wolbachia* in *W. bancrofti*, two separate sets of primers were used and PCR amplifications were done keeping all other conditions constant. Figure 1 shows the agarose gel electrophoresis of PCR products obtained by amplification of a putatively small amount of *Wolbachia* DNA, using 16 S rRNA primers as well as WSP primers. Amplification of *Wolbachia*-specific genes yielded distinct bands at 207 bp (16 S rRNA) and 590 bp (WSP) confirming the presence of the endosymbiont *Wolbachia* in *W. bancrofti.* Out of the 39 and 44 samples studied (Bankura and Birbhum, respectively), 35 and 40, respectively, were found to be positive and the remaining 4 in both the groups yielded no bands. Thus, the number of positive persons (75) represents a statistically significant

population (*Z*-test) based on the prevalence of filaria in the region. The amplification of *E. coli* DNA using 16 S rRNA primers (EUBAC-1 and EUBAC-2) yielded a distinct band at 1.5 kb whereas no amplification was recorded when *Wolbachia*-specific primers (FIL-5 and FIL-6) were used (figure 1). This shows the specificity of the PCR protocol used in the present study for detecting the filarial endosymbiont *Wolbachia*.

Electron micrographs of longitudinal sections of mf reveal the presence of *Wolbachia* in the hypodermal cells (figure 2). The bacterial entities were observed within a single cell where they were separated from each other by very thin strands of hypodermal cytoplasm and vacuolar membranes. They were generally ovoid in shape and had a granular appearance. The presence of *Wolbachia* was demonstrated in all the mf samples studied.

4. D\iscussion

Studies on different filarial nematodes and their endosymbiont, *Wolbachia*, indicate a long-term and stable association between the two organisms (Taylor *et al*. 1999). The obligatory symbiotic relationship is evident from several reports on filarial worms, where elimination of *Wolbachia* by treatment with tetracycline/doxycycline decreases host fitness in several non-specific ways including survival, developmental success and reproduction (Hoerauf *et al.* 1999). On the contrary, tetracycline shows no effect on the filarial worm *Acanthocheilonema viteae*, which does not have a symbiotic relationship with *Wolbachia* (Hoerauf *et al*. 1999). In the present study, the presence of *Wolbachia* in *W. bancrofti* has been shown by electron microscopic study of mf and PCR for the *Wolbachia*-specific 16 S rRNA and WSP genes. Thus, an obligatory symbiotic relationship is evident from the molecular and ultrastructural analysis of the *Wolbachia* endosymbiont and *W. bancrofti* collected from two different districts of West Bengal, i.e. Bankura and Birbhum. The presence of *Wolbachia* in a large number of patients suffering from bancroftian filariasis in the two districts of West Bengal shows the indispensable association between the filarial worm and the endosymbiont. In future, it would be interesting to study a larger section of the population for the prevalence of *W. bancrofti* as well as its endosymbiont *Wolbachia.* The findings may help in the formulation of an effective eradication programme.

Understanding this mutualistic/symbiotic relationship (for reproduction and viability) between the bacteria and the filariae that harbour them may eventually lead to the discovery of new drug targets.

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