DETECTION AND CHARACTERIZATION OF WOLBACHIA INFECTIONS IN WUCHERERIA BANCROFTI (SPIRURIDA: ONCHOCERCIDAE) VAR. PACIFICA AND Aedes (Stegomyia) polynesiensis (Diptera: Culicidae)

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Abstract. Despite control programs based on mass drug administration (MDA) of microfilaricidal compounds, Bancroftian lymphatic filariasis remains a problem in French Polynesia. For an alternative strategy to MDA, we investigated the potential role of Wolbachia to control filarial transmission. Wolbachia are intracellular α-proteobacteria endosymbionts that infect a broad range of insects and nematodes. These bacteria have a suspected role in the pathogenesis of filariasis. They also may be useful in mosquito control through cytoplasmic incompatibility. To detect and characterize these bacteria in the filarial and mosquito-vectors in French Polynesia, a survey was conducted on field-collected mosquitoes and microfilariae from infected people. Samples were analyzed by a polymerase chain reaction and gene sequencing. The results indicate that these bacteria are widespread. Sequence analysis of the wsp and ftsZ genes positioned the Aedes polynesiensis Wolbachia in cluster A and Wuchereria bancrofti var. pacifica Wolbachia in cluster D. The implications for possible improved treatment and vector control are discussed.

INTRODUCTION

Wolbachia bacterial endosymbionts are maternally inherited intracellular α-proteobacteria that infect a broad range of invertebrate host including insects and filarial nematodes.1,2 They play an important role in the development and pathogenesis of the filariae Onchocerca volvulus, Brugia malayi, and Wuchereria bancrofti. Inflammatory reactions are a major pathologic symptom of filarial infections and the Wolbachia symbionts of filarial nematodes appear to contribute to the inflammation process.3–8

In view of the importance of Wolbachia endosymbionts for the long-term survival and the development of lymphatic filarial nematodes as demonstrated by the effects of anti-Wolbachia chemotherapy on worms in animal models and in vitro,9–12 a new chemotherapeutic strategy using the tetracycline class compounds that target these endosymbionts has been envisaged for the treatment of human filariasis.13 Wolbachia was previously detected in nocturnally periodic W. bancrofti microfilariae.14–16 The presence of Wolbachia in the diurnally subperiodic Polynesian strain of filarial W. bancrofti variety pacifica,17,18 has not been described. Before we can plan a therapeutic trial targeting Wolbachia with a tetracycline class antibiotic treatment, we need to assess the prevalence of Wolbachia endosymbionts in microfilariae from infected patients in French Polynesia.

In arthropods, Wolbachia infections are associated with numerous host reproductive alterations, including cytoplasmic incompatibility.19,20 This alteration causes a reduction in the successful egg-hatch rate in crosses between individuals infected with different Wolbachia strains. Infected males mating with females that are either uninfected or harbor a different infection type results in infertility.21 Strategies based on cytoplasmic incompatibility have been proposed for both the suppression and replacement of host populations. It is crucial to know which Wolbachia strain(s) is or are present in natural mosquito populations before any plan to use cytoplasmic incompatibility to reduce transmission by the vector can be undertaken.

In this report, we describe a study using a polymerase chain reaction (PCR) and gene sequencing to detect and characterize Wolbachia bacteria for two different purposes. First, microfilariae from 22 patients from different islands in archipelagos highly endemic for Bancroftian lymphatic filariasis were analyzed for Wolbachia as a prerequisite to planning a therapeutic trial with antibiotics known to affect filarial development. Second, we conducted an initial survey for Wolbachia infection in the mosquito Aedes polynesiensis, the main vector for lymphatic filariasis in French Polynesia, to describe the geographic distribution and eventual polymorphism in Wolbachia strains with the goal of exploring the possibility of using cytoplasmic incompatibility as a transmission control strategy.

MATERIALS AND METHODS

Blood sampling. The protocol for this study was reviewed and approved by the Ethics Committee of French Polynesia. Before taking a blood sample, each microfilaremic volunteer was read a consent form and required to sign it. Patients from different islands in the Society and Marquesas archipelagos were sampled to recover W. bancrofti microfilariae. Blood samples (4 mL) were collected from the left arm (after it was cleansed with 70% isopropanol) into EDTA-Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ). Volunteers with high levels of microfilariae were asked to donate a second blood sample of 50 mL.

Counting of microfilariae. Microfilariae were concentrated by membrane filtration (3-μm pore membrane; Nuclepore Corp., Pleasanton, CA) of 1 mL of venous blood diluted two-fold with 1 mL of NaHCO3 solution (2 g/L). Filters were then stained with Giemsa prior to microscopic examination and enumeration.22–23 The remaining blood sample was first diluted with saline solution before carefully filtering (5–20 mL per filter). Filters were incubated in a saline solution for two hours at room temperature to enable microfilariae to release from the filter. Samples were then centrifuged for five minutes at 1,500 × g and the supernatant was discarded. Microfilariae pellets in 0.5 mL of saline solution were stored at −80°C until use.

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Field-collected mosquitoes. Since *Ae. polynesiensis* cannot be captured in adequate numbers with any commercially available trap, mosquitoes were collected in human landing catches by aspiration as they landed on the collector. Captured mosquitoes were placed in labeled, cotton-stoppered test tubes and held at 5–8°C in an ice box until transported to the laboratory. In the laboratory, they were desiccated for three hours at 90°C in pools of 20. The mosquito pools were stored at room temperature until used.

Laboratory-reared mosquitoes. Laboratory mosquito colonies were started from larvae collected from natural breeding sites (crab burrows, small ponds, and puddles). Larvae were reared in trays filled with one liter of distilled water and fed powdered cat pellets. The resulting adults were reared in 30-cm³ cages. Cages were kept in an environmental chamber at a temperature of 27–29°C and a relative humidity of 80–90% on a 16:8-hour light:dark cycle. Adult mosquitoes had access to a cotton wick soaked in a 10% sucrose solution. Eggs to maintain the colony were obtained by feeding mosquitoes on laboratory rats.

Tetracycline treatment of laboratory-reared mosquitoes. First attempts to remove *Wolbachia* from *Ae. polynesiensis* by treating larvae were unsuccessful due to high mortality. Thereafter, young adults (G₁) were used. At days 2 and 3 after emergence, tetracycline (ref. T-3383, final concentration 0.25 mg/mL; Sigma, St. Louis, MO) was added to the 10% sucrose solution used for feeding mosquitoes. Five-day-old females were allowed to blood feed and eggs were collected for colony maintenance. The subsequent two generations of adults (G₂ and G₃) were again treated with the 0.25 mg/mL tetracycline sucrose solution. The G₁, G₂, and G₃ adult females (n = 6 of each generation) were dissected and whole abdomens or ovaries were tested for *Wolbachia*.

Extraction of DNA. Extraction of DNA was done using the DNeasy Tissue Kit (catalog no. 69504; Qiagen, Hilden, Germany) according to the manufacturer’s protocol for animal tissues for both *microfilariae* (2,000–100,000) and individual mosquito tissues (whole abdomen or ovaries). Mosquito pools were extracted as previously described.

Screening for Wolbachia DNA by polymerase chain reaction. Screening for *Wolbachia* endobacteria DNA was done with a PCR using the specific primers 16S Wolb F and 16S Wolb R, as previously described. Amplification was done with a Mastercycler gradient PCR thermocycler (Eppendorf AG, Hamburg, Germany) using Hot Start *Taq* polymerase (Qiagen) and 1 or 5 µL of DNA sample in a reaction volume of 25 µL. Samples were incubated at 94°C for 15 minutes, followed by 38 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 3 minutes. Amplification products were then incubated at 72°C for 10 minutes. The reaction mixture contained 1 unit of *Taq* polymerase, 10 pmols of both primers, 5 mM of each dideoxynucleotide, and 2 µL of 25 mM MgCl₂ in 1× PCR buffer (Qiagen). A negative control for the PCR assay (sterile distilled water instead of DNA extract in the reaction mixture) was included in each run. Analysis of the PCR products was conducted by gel electrophoresis. Ten microliters of the PCR product was loaded onto a 1.5% agarose gel containing ethidium bromide (0.5 µg/mL) for visualization of the amplicon bands (approximately 1,000 base-pairs) with ultraviolet light.

Sequencing of Wolbachia DNA. Amplification were performed using 5 µL of DNA extract in reaction volumes of 25 µL containing 1× buffer (Qiagen), 2 mM MgCl₂, 0.2 mM of each dideoxynucleotide, 0.4 µM each of forward and reverse primers and 1 unit of *Taq* polymerase (Qiagen). The *Wolbachia* wsp gene was amplified by PCR using primers wsp int F and wsp int R for filarial material. Primers for specific amplification of A group (136F/691R) and B group (81F/522R) wsp were used for mosquito extracts. The *Wolbachia* ftsZ gene was amplified by PCR using primers ftsZ uni/F/uniR, F1/R1, and F213 (5'-CAAAAGCAGCCAGAGAAG-3') and R500 (5'-TAATAAGCCCTGGCATTGAC-3').

The thermal profile used for Wspint F/Wspint R and Wsp136F/Wsp691 R was 94°C for 15 minutes; 35 cycles at 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 90 seconds; and 72°C for 10 minutes. For Wsp 81 F/Wsp 522 R, the thermal conditions were the same, except that the annealing temperature was 55°C. The thermal profile used for ftsZ uni/F/uniR, F1/R1, and ftsZ F213/R500 was 94°C for 15 minutes; 38 cycles at 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 3 minutes; and 72°C for 10 minutes. For ftsZ F1/R1, the thermal conditions were the same, except that the annealing temperature was 47°C.

The PCR products were gel purified using the Qiaquick Gel Extraction kit (Catalog no. 28704; Qiagen). Sequencing reactions were conducted using the Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer-Applied Biosystems, Inc., Warrington, United Kingdom). Unincorporated dideoxy terminators were removed using DyeEx Spin columns (Qiagen). The products were sequenced in an automated ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). Both strands of the PCR products were sequenced.

Sequences of the 22 Polynesian microfilarial DNA extracts were aligned and compared with the prealigned wsp and ftsZ sequences (GenBank accession no. AJ252180 and AF081198) of previously published periodic *W. bancrofti*. Sequences for the six *Ae. polynesiensis* DNA extracts were aligned and compared with the prealigned wsp (AF020058, AF317475, AF317476, AF317477, AF317380, AF317383, AF317484, AF317385, AF317388, AF317390, and AY553501) and ftsZ (U28206 and U28209) sequences registered for mosquito *Wolbachia*. The new sequence obtained in this study was deposited in GenBank (accession no. AY988806).

RESULTS

PCR screening of Wolbachia DNA in *Wuchereria bancrofti* var. *pacificia microfilariae*. Twenty-two people from different islands in the Society and Marquesas archipelagoes participated in the study. All DNA extracts of the microfilariae samples contained *Wolbachia* DNA (samples from treated as well as untreated people). Positive samples were from Tahiti (1), Moorea (2), Raiatea (9), Tahaa (2), Huahine (5), Bora-Bora (1), and Fatu-Hiva (2).

Sequencing of *Wolbachia* endosymbiont DNA in *Wuchereria bancrofti* var. *pacificia*. The microfilarial DNA extract of one patient from each screened island was analyzed. The wsp int and ftsZ primers gave amplification products of 588 and 1011 basepairs, respectively. Gene fragments were sequenced directly and the sequences were aligned to the wsp and ftsZ genes available for *W. bancrofti* *Wolbachia* (European Molecular Biology Data Library, Heidelberg, Germany; acces-
Bioassay numbers AJ252180 and AF081198, respectively). Analysis showed that all six *pacific* isolates contained an identical Wolbachia type, similar to the D-type Wolbachia for *W. bancrofti* from Sri Lanka and Papua New Guinea.

**PCR screening for Wolbachia DNA in Ae. polynesiensis mosquitoes.** Mosquito samples of four laboratory-reared colonies, as well as pools of field-collected mosquitoes, were found to contain Wolbachia DNA. The tetracycline-treated line of the Tahiti-Atimaono colony was cleared of Wolbachia infections at rates of 66% for G2 adults and 99% for G3 adults. The data indicate that Wolbachia infection is widespread in the screened areas (Table 1).

**Sequencing of Wolbachia endosymbiont DNA in Ae. polynesiensis mosquitoes.** One DNA extract from each of the screened islands (Tahiti-Atimaono, Moorea-Afareaitu, Raitatea-Tevaitoa, Maupiti island, Tahuata-Vaitahu, and Fatu-Hiva-Hanavavae) was used. For the wsp gene, only the A group-specific primers yielded fragments of 594 basepairs. For the ftsZ gene primers, a 1016-basepair product was obtained.

All sequences obtained (wsp and ftsZ genes) were identical in the six isolates/strains and were grouped in the A clade. The wsp sequence was identical to the registered sequence AY 535013, and the ftsZ sequence obtained was deposited in GenBank under the accession number AY898806.

**DISCUSSION**

Wolbachia endosymbionts are found in many filarial species infecting animals and humans, including *W. bancrofti*. They have been detected in *W. bancrofti* microfilariae from 2 individuals in Papua New Guinea, 2 in Sri Lanka, and from different filarial-endemic locations in India, as well in 93 patients in a therapeutic trial in 6 villages in western Ghana. In these areas, a nocturnally periodic form of Bancroftian lymphatic filariasis was studied. For the Ghanian patients with lymphatic filariasis, Wolbachia depletion by doxycycline treatment led to a sustained reduction of microfilaraemia after 12 months if given alone, and to a complete absence of microfilaraemia when followed by a single dose of ivermectin.

In French Polynesia, a subperiodic form of Bancroftian lymphatic filariasis is found and the filarial isolates/strains involved are reported as the *pacific* variety of *W. bancrofti*. Control programs used diethylcarbamazine (6 mg/kg of body weight) from 1993 to 1998. This treatment regimen was changed to a combination of diethylcarbamazine (6 mg/kg of body weight) and albendazole (400 mg/person) from 2000 to 2005. Although some success in reducing the prevalence of infection has been observed, filariasis remains a problem in both the Marquesas and Society archipelagos. The results of our PCR survey for Wolbachia in *W. bancrofti* var. pacifica microfilariae suggest that Wolbachia infection is widespread in this area. Sequencing the wsp and ftsZ genes showed that all samples contained an identical Wolbachia type, similar to the D type Wolbachia identified in *W. bancrofti* microfilariae from Sri Lanka and Papua New Guinea.

The maternally inherited bacterial endosymbiont Wolbachia is widespread among arthropods. The diversity of Wolbachia infection in mosquito taxa has been investigated in Southeast Asia, Europe and Africa, and the Pacific region. Reproductive parasitism of Wolbachia in arthropods is an interesting approach to the control of mosquito vectors because the symbiont can reduce the fitness of those individuals that do not contain the symbiont or contain another strain of symbiont. To enhance the prospects of successfully reducing mosquito populations, the type of Wolbachia strains already present in natural mosquito populations must be determined before mosquitoes with a Wolbachia strain are released.

In French Polynesia, *Ae. polynesiensis* is a secondary dengue vector, as well as the main vector of lymphatic filariasis caused by subperiodic *W. bancrofti*. Despite multiple mass drug administration (MDA) to eliminate the parasite, filariasis remains endemic in French Polynesia because of 1) the year-round relatively high mosquito population densities of the day-biting *Ae. polynesiensis* vector and 2) the high efficiency of *Ae. polynesiensis* in developing infective larvae in the vector.

In certain filaria-mosquito combinations, the number of infective larvae (third-stage L3) that develop in a mosquito is not proportional to the number of microfilariae ingested by that mosquito. In mosquitoes such as *Anopheles*, as the number of microfilariae ingested increases, the yield of L3 per microfilaria increases, a process known as facilitation. Each

<table>
<thead>
<tr>
<th>Island Type Result</th>
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<tbody>
<tr>
<td>Tahiti-Atimaono colony</td>
</tr>
<tr>
<td>Abdomen, female n = 20</td>
</tr>
<tr>
<td>20/20+</td>
</tr>
<tr>
<td>Gravid ovaries n = 20</td>
</tr>
<tr>
<td>20/20+</td>
</tr>
<tr>
<td>Non gravid ovaries n = 5</td>
</tr>
<tr>
<td>5/5+</td>
</tr>
<tr>
<td>Tetracycline-treated female*</td>
</tr>
<tr>
<td>n= 18</td>
</tr>
<tr>
<td><strong>G1</strong>: 6/6+</td>
</tr>
<tr>
<td><strong>G2</strong>: 2/6+</td>
</tr>
<tr>
<td><strong>G3</strong>: 0/6+</td>
</tr>
<tr>
<td>Raiatea-Tevaitoa 2000 n = 2 2/2+</td>
</tr>
<tr>
<td>Raiatea-Opoa 1998 n = 2 2/2+</td>
</tr>
<tr>
<td>Maupiti 1999 n = 1 +</td>
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<tr>
<td>Maupiti 1999 n = 1 +</td>
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<tr>
<td>Tahuata-Vaitahu 2000 n = 1 +</td>
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<td>Tahuata-Hanatena 2000 n = 1 +</td>
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<td>Tahuata-Motopu 2003 n = 1 +</td>
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<td>Tahuata-Haptoni 2003 n = 1 +</td>
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<tr>
<td>Fatu Hiva-Omoa 1996 n = 1 +</td>
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<tr>
<td>Fatu Hiva-Hanavavae 1998 n = 1 +</td>
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* Whole abdomen or dissected ovaries of tetracycline-treated females.
The presence of a Wolbachia endosymbiont was confirmed in diurnally subperiodic W. bancrofti for the first time. The Wolbachia type was identical to that found in the nocturnally periodic W. bancrofti. Given the encouraging results observed in antibiotic treatment of Bancroftian lymphatic filariasis in Ghana, it may be useful to assess the efficiency of adding antibiotic treatment to the current antifilarial treatment regimen in a therapeutic trial with microfilariae-infected patients in French Polynesia.

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