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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.[1,14–16] 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 archipelagoes highly endemic for Bancroftian lymphatic filariasis were analyzed for *Wolbachia* as a prerequisite to planing 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 Ethic's 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 archipelagoes were sampled to recover *W. bancrofti* microfilariae. Blood samples (4 mL) were collected from the left arm (after it was cleaned 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 $NaHCO_3$ 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. polynesiesis* cannot be captured in adequate numbers with any commercially available trap, mosquitoes were collected in human landing catches[24] 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-cm^3 cages.[25] 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 (G_1) 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 (G_2 and G_3) were again treated with the 0.25 mg/mL tetracycline sucrose solution. The G_1, G_2, and G_3 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 DNAeasy 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.[26]

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.[27] 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 pmoles of both primers, 5 nM of each dideoxynucleotide, and 2 μL of 25 mM $MgCl_2$ 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 basepairs) 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 $MgCl_2$, 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.[1] Primers for specific amplification of A group (136F/691R) and B group (81F/522R) wsp were used for mosquito extracts.[28] The *Wolbachia ftsZ* gene was amplified by PCR using primers ftsZ uniF/uniR, F1/R1, and F213 (5′-CAAAAGCAGCCAGAGAAG-3′) and R500 (5′-TAATAAGCCCTGGCATGAC-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 uniF/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 for the 22 Polynesian microfilarial DNA extracts were aligned and compared with the preligned *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 preligned *wsp* (AF020058, AF317475, AF317476, AF317477, AF317380, AF317383, AF317484, AF317385, AF317388, AF317390, and AY535013)[28–30] and *ftsZ* (U28206 and U28209)[31] sequences registered for mosquito *Wolbachia*. The new sequence obtained in this study was deposited in GenBank (accession no. AY898806).

RESULTS

PCR screening of *Wolbachia* DNA in *Wuchereria bancrofti* var. *pacifica* 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* endosymbiot DNA in *Wuchereria bancrofti* var. *pacifica*. 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-

sion numbers AJ252180 and AF081198, respectively). Analysis showed that all six *pacifica* 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 G_2 adults and 99% for G_3 adults. The data indicate that *Wolbachia* infection is widespread in the screened areas (Table 1).

Sequencing of *Wolbachia* endosymbiot DNA in *Ae. polynesiensis* mosquitoes. One DNA extract from each of the screened islands (Tahiti-Atimaono, Moorea-Afareaitu, Raiatea-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,[31] 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*.[3] They have been detected in *W. bancrofti* microfilariae from 2 individuals in Papua New Guinea, 2 in Sri Lanka,[1,14] 20 from different filarial-endemic locations in India,[15] as well in 93 patients in a therapeutic trial in 6 villages in western Ghana.[16] In these areas, a nocturnally periodic form of Bancroftian lymphatic filariasis was studied. For the Ghanean patients with lymphatic filariasis, *Wolbachia* depletion by doxycycline treatment led to a sustained reduction of microfilaremia after 12 months if given alone, and to a complete absence of microfilaremia when followed by a single dose of ivermectin.[16]

In French Polynesia, a subperiodic form of Bancroftian lymphatic filariasis is found and the filarial isolates/strains involved are reported as the *pacifica* 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.[1]

The maternally inherited bacterial endosymbiont *Wolbachia* is widespread among arthropods.[32] The diversity of *Wolbachia* infection in mosquito taxa has been investigated in southeast Asia,[33,34] Europe and Africa,[35] North America,[36] and the Pacific region.[30] 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[35] or contain another strain of symbiont.[21,30] 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.[37]

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.[38] Each

TABLE 1
Area and year of collection of *Aedes polynesiensis* mosquitoes screened for *Wolbachia* endosymbiont

Screening of laboratory-reared mosquitoes			Screening of pools of field-collected mosquitoes			
Island	Type	Result	Island	Year of collection	Number tested	Result
Tahiti-Atimaono 2000 colony	Abdomen, female n = 20	20/20+	Raiatea-Tevaitoa	2000	n = 2	2/2+
	Gravid ovaries n = 20	20/20+	Raiatea-Opoa	1998	n = 2	2/2+
	Non gravid ovaries n = 5	5/5+	Maupiti	1999	n = 1	+
	Tetracycline-treated female* n = 18	G1: 6/6+		2002	n = 2	2/2+
		G2: 2/6+	Maupiti	1999	n = 1	+
		G3: 0/6+	Tahuata-Vaitahu	2000	n = 1	+
Moorea-Afareaitu 2001 colony	Abdomen, female n = 3	3/3+	Tahuata-Hanatetena	2000	n = 1	+
Raiatea-Tevaitoa 2003 colony	Abdomen, female n = 3	3/3+		2003	n = 1	+
Tahuata-Vaitahu 2003 colony	Abdomen, female n = 3	3/3+	Tahuata-Motopu	2000	n = 1	+
				2003	n = 1	+
			Tahuata-Hapatoni	2003	n = 1	+
			Fatu Hiva-Omoa	1996	n = 1	+
				1998	n = 1	+
			Fatu hiva-Hanavavae	1998	n = 1	+

* Whole abdomen or dissected ovaries of tetracycline-treated females.

ingested microfilaria that is successful in reaching the hemocele increases the permeability of the stomach wall for the next microfilaria. In contrast, limitation, which is exhibited in some culicine mosquitoes, especially *Ae. polynesiensis*,[38,39] is the process by which the mosquito becomes relatively more efficient as it ingests fewer microfilariae. This phenomenon makes the interruption of filarial transmission particularly difficult because large outbreaks of lymphatic filariasis may develop from just a few residual cases. This is why vector control may be essential to eliminate filariasis in areas where *Aedes* are the vectors. The present control techniques need to be improved and optimized, especially for the difficult-to-control *Ae. polynesiensis* mosquito. In this context, the reproductive endosymbiont, *Wolbachia,* offers more than a glimmer of hope.

The results of our PCR survey for *Wolbachia* in *Ae. polynesiensis* suggest that infection with this bacteria is widespread in mosquito populations. Sequencing the *wsp* and *ftsZ* genes showed that all samples screened contained an identical *Wolbachia* type from the A clade. Our data, which are similar to those data recently reported,[31] suggest that polymorphism of the *Wolbachia* infection is not present in the area screened in our study. Based upon these results, we are encouraged to develop both cytoplasmic incompatibility as a strategy for vector control and the use of tetracycline therapy in Polynesia for integration with the present MDA using diethylcarbamazine and albendazole to stop filarial transmission.

In conclusion, 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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