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Rate of Elimination of *Wolbachia pipientis* by Doxycycline In Vitro Increases following Drug Withdrawal

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***Wolbachia pipientis* is an obligate intracellular bacterium within the family *Anaplasmataceae* that infects many terrestrial arthropods and arthropod-transmitted nematodes (filariae). Several filarial species are major human pathogens, and antibiotics with activity against *Wolbachia* offer a promising new therapeutic approach, since the adult worms are relatively refractory to conventional anthelmintics but depend on *Wolbachia* for reproduction and viability. In a natural filarial parasite of cattle, *Onchocerca ochengi*, intermittent chemotherapy is adulticidal whereas the equivalent dose administered as a continuous treatment is not. To investigate this further and to aid the design of efficacious regimens for human therapy, we used *Wolbachia*-infected *Aedes albopictus* mosquito cells in vitro. Here, we describe for the first time the accelerated depletion of bacteria after antibiotic withdrawal relative to the rate of elimination in the continuous presence of the drug. Mosquito cells were incubated with doxycycline while changes in 16S (bacterial) and 18S (host) rRNA and rRNA genes were determined by quantitative PCR assays. In cultures treated for 7 or 14 days followed by 7 days of drug withdrawal, the *Wolbachia*-to-*Aedes* rRNA ratio declined by ~6 log, whereas immediately after 14 or 21 days of continuous treatment, the reduction was only ~4 log ($P < 0.05$). However, low levels of 16S rRNA remained after 21 days of treatment, irrespective of whether doxycycline was withdrawn. Application of similar methodology to related intracellular bacteria may reveal that this posttreatment effect is not restricted to *Wolbachia* and could have wider implications for the design of intermittent regimens for antibiotic chemotherapy.**

Wolbachia pipientis is an obligate intracellular bacterium within the order *Rickettsiales* and the family *Anaplasmataceae* (14) which infects a wide variety of arthropod taxa and is transmitted predominantly by the vertical route (39). In 1995 (38), the intracellular bacteria described 20 years earlier from several filarial nematode parasites (27) were identified as members of *Wolbachia*, and the acquisition of these organisms by the filariae is proposed to have occurred approximately 100 million years ago, via horizontal transfer from hematophagous arthropod vectors (4). *Wolbachia* spp. have been divided into six supergroups comprising A, B, E, and F in arthropods (4, 26) and C and D in filariae (4), but despite significant differences between the species at the 16S rRNA gene level, *W. pipientis* is the only formally recognized member of the genus (14).

The major filarial diseases of humans, lymphatic filariasis and onchocerciasis (river blindness), are responsible for a combined global morbidity of 6.5 million disability-adjusted life years (35), and control and eradication strategies are constrained by the refractoriness of adult filariae to anthelmintics, such as ivermectin (7) and diethylcarbamazine (29). However, new opportunities for the adulticidal therapy of filarial diseases have arisen from the identification of *Wolbachia* in the etiological agents of both lymphatic filariasis (*Wuchereria bancrofti* and *Brugia malayi* [4]) and onchocerciasis (*Onchocerca volvulus* [18]), although *Loa loa* (African eye worm) lacks these bacteria (8). Studies of *Wolbachia*-positive filarial parasites of animals revealed that tetracyclines induced growth retardation, inhibition of embryogenesis (5, 22), and, in the case of *Onchocerca ochengi*, a parasite of cattle, killing of adult worms (25), suggesting a dependent relationship between the bacteria and the filariae that harbor them. Conversely, the *Wolbachia*-negative filaria *Acanthocheilonema viteae* was not affected by tetracycline treatment (22). Subsequent human field trials have demonstrated that 6 weeks of daily doxycycline (DOX) therapy leads to chronic sterilization of adult female *O. volvulus*, preventing development of the pathogenic microfilarial stage of the parasite (21, 23), and that 8 weeks of daily DOX has significant adulticidal activity against *Wuchereria bancrofti* (40).

The original report (25) of unequivocal adulticidal activity of oxytetracycline against a filarial nematode was an in vivo trial using *O. ochengi* of cattle, a natural parasite of bovines that represents the closest extant relative of *O. volvulus* (46) and an excellent model for chemotherapeutic and immunological investigations (1). In a follow-up study (16), the intermittent delivery of oxytetracycline was found to be more effective than a shorter-term, continuous administration of the same total dose: a regimen comprising monthly injections for 6 months killed >60% of adult female worms, while daily treatment for 14 days failed to have any substantive effect. Analysis of *Wolbachia* density revealed that depopulation of the organisms after the short-term intensive therapy was considerable but transient, with recrudescence of bacteria to pretreatment levels by 24 weeks after the first dose. In contrast, prolonged intermittent regimens induced a sustained depletion of *Wolbachia* that resulted in worm death by 52 weeks posttreatment.

These results are unusual in that concentrations of oxytetracycline above the MIC for *W. pipientis*, which has been estimated from an in vitro study as 1 to 4 μg/ml (19), could not have been maintained in cattle tissues during intervals of 1 month, even considering the long-acting formulation used for intramuscular delivery (41). Therefore, we sought to investi-

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TABLE 1. Oligonucleotide primers used for quantitative PCR

Target	Orientation	Nucleotide sequence (5′→3′)	Amplicon length (bases)	GenBank accession no. (reference)
Aedes albopictus 18S rRNA and rRNA genes	Sense	CCGTGATGCCCTTAGATGTT	100	X57172 (3)
	Antisense	ATGCGCATTTAAGCGATTTC		
Wolbachia pipientis 16S rRNA and rRNA genes	Sense	TTGCTATTAGATGAGCCTATATTAG	99	X61767 (31)
	Antisense	GTGTGGCTGATCATCCTCT		

gate the dynamics of *Wolbachia* elimination in vitro using a cell line (Aa23) derived from the mosquito *Aedes albopictus* that is stably infected with a *Wolbachia* strain from the B supergroup (32). This insect cell line was selected because to date, only short-term primary cultures of filarial cells have been maintained in vitro, precluding analysis of antibiotic action (20). Furthermore, Aa23 has been used to determine the sensitivity of *Wolbachia* to a wide range of antibiotics, and in agreement with in vitro and in vivo studies of filariae (34, 42, 44), only the tetracyclines and rifampin were found to have significant activity (15, 19). Here, we present a quantitative analysis of *Wolbachia* 16S rRNA and rRNA gene levels during and after DOX treatment as determined by real-time PCR.

MATERIALS AND METHODS

Cell culture and antibiotic treatment. The Aa23 cells were incubated at 26°C in 25-cm^2 culture flasks containing 5 ml of medium comprising 45% (vol/vol) Mitsuhashi-Maramorosch medium (Promocell, Heidelberg, Germany), 45% (vol/vol) Schneider's *Drosophila* medium (Promocell), 10% (vol/vol) fetal calf serum (Cambrex Bio Science, Walkersville, Md.), and 0.18% (wt/vol) L-glutamine (Sigma-Aldrich, St. Louis, Mo.). For subculture, the confluent cell monolayer was dislodged with a cell scraper every 7 days, and 20% of the suspension was divided into each new flask. To prepare stock DOX solution (5 mg/ml), solid DOX hyclate (Fluka, Buchs, Switzerland) was dissolved in ultrapure water, filter sterilized, and stored in aliquots at −20°C. This was applied to the cells at a final concentration of 0.25 μg/ml (the minimal bactericidal concentration for *W. pipientis* as determined by Hermans et al. [19]), and treatment continued for 3, 7, 14, or 21 days, with full replenishment of DOX after 4 days and subculture (and addition of fresh antibiotic) every 7 days. For each group, four replicate flasks were harvested immediately after the end of treatment, while four replicate subcultures were maintained without DOX for 7 days (posttreatment phase) prior to harvesting. Pretreatment data were obtained from parallel cultures that had not been exposed to any antibiotics.

Nucleic acid extraction and synthesis of cDNA. Cells were scraped from each flask, centrifuged at 150 × *g* for 10 min, and mechanically homogenized in 1 ml of TRI Reagent (Sigma). Following centrifugation at 12,000 × *g* (4°C) for 10 min, insoluble material was discarded, and total RNA was isolated in parallel with genomic DNA according to the manufacturer's instructions. Genomic DNA was dissolved in 0.6 ml 8 mM sodium hydroxide and stored at 4°C, while RNA was dissolved in 20 μl diethyl pyrocarbonate-treated water by incubation at 55°C for 10 min. For digestion of contaminating genomic DNA, 16 μl of RNA solution was incubated with 2 units of DNase I (amplification grade; Invitrogen, Carlsbad, Calif.) for 15 min at room temperature; this was inactivated by 15 min of incubation at 65°C in the presence of 2.5 mM EDTA. Hybridization with 1 μg random hexadeoxynucleotides (Promega, Madison, Wis.) was conducted at 70°C for 5 min, followed by 5 min on ice. Samples were divided into two aliquots for either cDNA synthesis or a reverse transcriptase-negative (RT$^-$) control; each reaction mixture contained 100 units of Moloney murine leukemia virus reverse transcriptase (RNase H$^-$ point mutant; Promega) or an equivalent volume of water, respectively, and 0.5 mM each deoxynucleoside triphosphate (Promega) in a final volume of 20 μl. Reaction conditions comprised an annealing step of 10 min at 25°C, an extension step of 60 min at 42°C, and a denaturation step of 10 min at 70°C. The resultant cDNA was stored at −20°C.

Quantitative (reverse transcriptase) PCR. Primers were designed to have equivalent predicted melting temperature using published sequence data (Table 1) and Primer3 freeware (36) and obtained by custom synthesis from http://www.biomers.net (Ulm, Germany). To prevent amplification of DNA from adventitious contaminants, the *W. pipientis* 16S amplicon was selected from a region of maximal divergence by alignment against 16S rRNA gene sequences from *Escherichia coli*, *Staphylococcus aureus*, and *Mycoplasma orale* using CLUSTALW freeware (http://clustalw.genome.jp). Quantitative DNA standards representing both full-length amplicons (Table 1) were synthesized by Sigma-Genosys (Haverhill, United Kingdom) as single-stranded oligonucleotides and diluted in a 10-fold series from 5×10^6 to 5×10^{-1} copies/μl in 100 ng/μl yeast tRNA (Invitrogen) to prevent aggregation. Reaction mixtures comprised 1× SensiMix (deoxyribosylthymine version; Quantace, Watford, United Kingdom), a pair of primers at 200 nM each, 1× SYBR green solution (Quantace), and 1 μl of DNA template in a final volume of 20 μl. Assays were performed on a DNA Engine Opticon 2 thermocycler (Bio-Rad Laboratories, Hercules, Calif.) under the following conditions: initial denaturation for 10 min at 95°C; 35 cycles at 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 15 seconds; and melting curve analysis between 50 and 95°C to confirm the specificity of the amplification products. Genomic DNA, cDNA, and RT$^-$ controls were assayed separately for *Wolbachia* or *Aedes* templates at two dilutions, while standard curves and no-template controls were included on every plate in duplicate. Copy numbers were obtained by linear regression analysis (interpolation), and the most accurate dilution was deemed to be that closest to the center of the standard curve or the greater dilution where inhibition was suspected. Although DNase treatment did not completely eliminate genomic DNA contamination from RNA, correction using the matched RT$^-$ controls indicated that the effect on *Wolbachia* 16S rRNA quantification was negligible, since genomic contamination never accounted for >2% of cDNA copy number and the typical level was <0.001%. Sensitivity was routinely <10 copies per reaction, and no-template controls were consistently negative.

Data processing and statistical analysis. Ratios of *Wolbachia* 16S rRNA to *Aedes* 18S rRNA and *Wolbachia* 16S rRNA genes to *Aedes* 18S rRNA genes were calculated by division of the respective copy numbers. Prior to data presentation and statistical analysis, ratios and unadjusted copy numbers for rRNA were normalized by $\log_{10}(x)$ transformation, while for rRNA genes, $\log_{10}(x + 1)$ transformation was applied to copy numbers and $\log_{10}(x + 10^{-5})$ to ratios, as zero values were present. Statistical analyses were performed in SPSS (SPSS Inc., Chicago, Ill.) using a univariate general linear model, with treatment period and posttreatment phase as fixed factors and the inclusion of an interaction term. Pairs of means within the treatment period were compared by Tukey's honestly significant difference test or Tamhane's T2 post hoc test as appropriate. The significance of mean differences by posttreatment phase at specific time points was analyzed by independent sample *t* tests. The critical probability (*P*) for statistical significance was <0.05 throughout.

RESULTS

Effect of DOX treatment on levels of *Wolbachia* 16S rRNA and rRNA genes. The copy numbers of *Wolbachia* 16S rRNA genes and rRNA were determined by quantitative (RT) PCR and compared between periods of DOX treatment ranging from 3 to 21 days, with or without an antibiotic withdrawal phase of 7 days. The decline in copy number of 16S rRNA was substantial and approximately linear, reaching a reduction of 6 orders of magnitude after 21 days (Fig. 1A). However, at no point did *Wolbachia* 16S rRNA become undetectable. After 3 days of treatment, the amount of 16S rRNA had already declined significantly compared to control levels ($P < 0.001$), and all subsequent reductions between consecutive time points were highly statistically significant ($P < 0.01$). The decline in

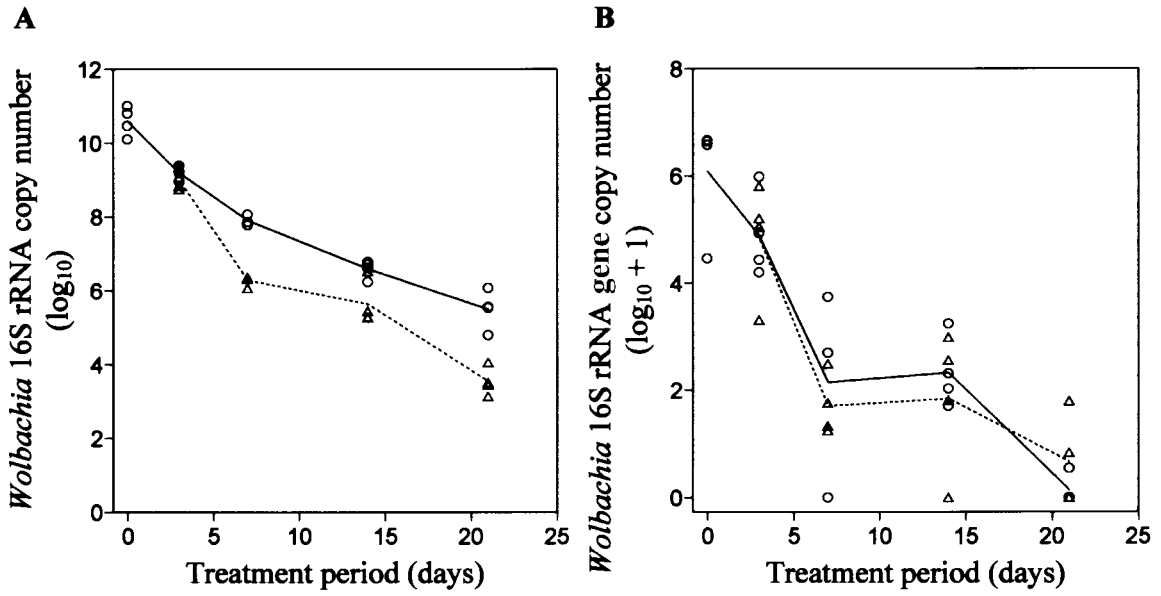

FIG. 1. Dynamics of depletion of *Wolbachia pipientis* by DOX in vitro as determined via quantification of 16S (A) rRNA and (B) rRNA gene copy numbers immediately after treatment (○) or following a 7-day posttreatment phase (△). Time on the *x* axis refers only to the duration of culture under continuous antibiotic treatment.

Wolbachia 16S rRNA gene copy number was more variable, but the scale of the reduction over 21 days was similar to that of 16S rRNA (Fig. 1B). At 7 days of treatment and subsequent time points, some replicates were negative for the 16S rRNA gene, although at 21 days, it was still detectable at extremely low copy numbers in a minority of replicates (Fig. 1B). The depletion of 16S rRNA gene copy number did not become statistically significant compared to that of the control until after 7 days of DOX treatment ($P < 0.001$), and a plateau was reached between 7 and 14 days, although there was a further significant decline between 14 and 21 days of treatment ($P = 0.019$) (Fig. 1B).

Effect of antibiotic withdrawal on levels of *Wolbachia* 16S rRNA and rRNA genes. When *Wolbachia* 16S rRNA copy number was quantified 7 days after withdrawal of DOX, a clear trend was apparent in terms of a reduction in 16S rRNA relative to the corresponding cultures that were not subjected to antibiotic withdrawal (Fig. 1A). This attrition was very highly statistically significant ($P < 0.001$) and furthermore, interacted significantly with the treatment period, indicating that the rate of decline in 16S rRNA levels was greater in cultures that underwent a posttreatment phase than in those that did not ($P < 0.001$) (Fig. 1A). However, no such effect was observed with 16S rRNA genes (Fig. 1B): neither the posttreatment phase per se nor its interaction with the treatment period was a statistically significant factor.

Effect of normalization of 16S rRNA and rRNA gene copy numbers against those of 18S rRNA and rRNA genes. Changes in *Aedes* (host cell) nucleic acid yield could represent alterations in cell number, size, or metabolism as well as reflecting variability in PCR inhibitors, RNases, extraction efficiency, and reverse transcription. Thus, *Aedes* 18S rRNA and rRNA genes were quantified alongside *Wolbachia* PCR templates, and 16S rRNA copy number was divided by 18S rRNA copies to produce a ratio; similarly, rRNA gene copies were also normalized (Fig. 2). The magnitude of the decline for both *Wolbachia* rRNA and rRNA gene levels, when expressed as ratios, was similar to that observed with the unadjusted copy numbers (compare Fig. 1 and 2). The statistical significance of the differences between consecutive time points was maintained for *Wolbachia* rRNA, except for comparisons between 7 and 14 days and 14 and 21 days. Furthermore, conversion to a ratio did not materially affect the statistically significant reductions that were apparent for *Wolbachia* rRNA gene copy numbers. Note that the rRNA gene ratio does not represent the number of bacteria per host cell, since *Wolbachia* has a single copy of the 16S rRNA gene (45) and *A. albopictus* cells contain several hundred copies of 18S rRNA genes per haploid genome (33). However, this ratio is still a useful indicator of changes in *Wolbachia* density resulting from treatment.

Effect of normalization on the interpretation of the posttreatment phase. The observed effect of antibiotic withdrawal in reducing *Wolbachia* 16S rRNA was more marked when visualized as a *Wolbachia*-to-*Aedes* rRNA ratio (Fig. 2A). The rapid rate of elimination of *Wolbachia* rRNA following withdrawal of DOX was clearly greater than that in the absence of the posttreatment phase ($P < 0.001$) (Fig. 2A). This could not be attributed simply to dilution of organisms in stasis by the additional subculture preceding the posttreatment phase, since the rRNA ratio was significantly lower for 7 days of treatment plus 7 days of withdrawal than for 14 days of treatment with no withdrawal (mean difference, 1.7 log; $P = 0.020$) (Fig. 2A). Moreover, a corresponding analysis for 14 days of treatment plus 7 days of withdrawal compared with 21 days of treatment and no withdrawal demonstrated an even greater disparity (mean difference, 2.1 log; $P = 0.004$) (Fig. 2A). The expression of rRNA gene levels as a *Wolbachia*-to-*Aedes* ratio revealed a significant effect of antibiotic withdrawal that had not been

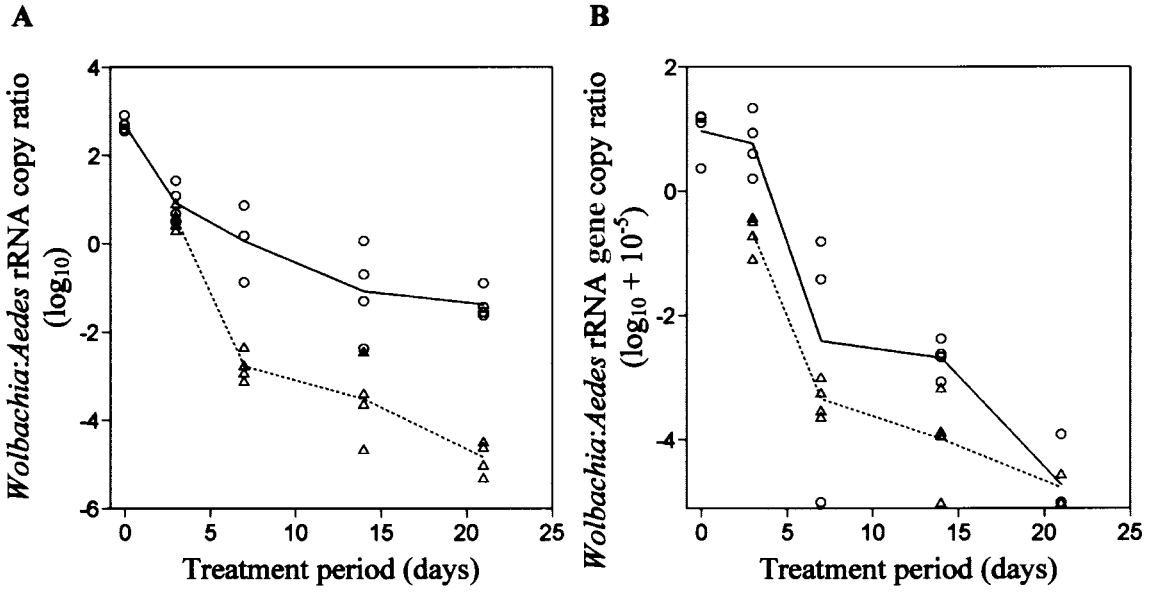

FIG. 2. Dynamics of depletion of *Wolbachia pipientis* by DOX in vitro as determined via quantification of *Wolbachia*-to-*Aedes* (A) rRNA and (B) rRNA gene copy ratios immediately after treatment (○) or following a 7-day posttreatment phase (△). Time on the *x* axis refers only to the duration of culture under continuous antibiotic treatment.

apparent with unadjusted copy numbers, and this mirrored the pattern for rRNA, with the posttreatment phase correlating with lower ratios ($P = 0.002$) (Fig. 2B). Accordingly, the rRNA gene ratio for 7 days of treatment with 7 days of withdrawal was significantly lower than that for 14 days of treatment and no withdrawal (mean difference, 0.7 log; $P = 0.018$) (Fig. 2B).

DISCUSSION

The amplification of rRNA to detect viable bacteria is a well-established method (13, 43) to overcome a key limitation of DNA-based assays, that is, spurious positive signals resulting from residual DNA in dead organisms (43). A major advantage of rRNA is that although it represents a constitutive, stable component of viable bacteria, it is rapidly degraded by periplasmic RNase I following damage to the cell membrane (12). However, since the rRNA content of bacterial cells is regulated by the rate of growth (28), rRNA gene levels were measured in parallel in the current study to aid interpretation of the RT-PCR data. To the best of our knowledge, this represents the first occasion in which both RNA and DNA have been quantified during antibiotic treatment of an obligate intracellular bacterium and builds upon previous analyses of *Wolbachia pipientis* in the Aa23 cell line, which used light microscopy (19) or quantitative PCR for genomic DNA only (15) to measure drug action.

The key finding of the present study was the marked increase in bacterial depletion that occurred after drug withdrawal, and this cannot be readily explained by previously reported phenomena, particularly when culture periods of equivalent lengths were specifically compared. Moreover, the consistency of the posttreatment effect for the *Wolbachia*-to-*Aedes* rRNA ratio was remarkable, occurring independently in four replicate flasks per group over three time points. In contrast to the postantibiotic and sub-MIC effects that have been widely reported for a broad range of drug/organism combinations, in which bacterial recovery is delayed after the antibiotic concentration has fallen below the MIC (30), the effect exhibited in our experiments extended to enhanced bactericidal activity and appears to be entirely novel. We did not wash host cells after drug withdrawal, since this would not remove DOX from intracellular reservoirs and furthermore, the in vivo situation is more closely mimicked by retaining sub-MIC levels of antibiotic in culture. Hence, DOX would have been present at a maximum concentration of 0.05 μg/ml (0.8 × MIC according to Hermans et al. [19]) at the beginning of the posttreatment phase, and this could account for lack of recrudescence but not the increased rate of bacterial depletion.

We can only speculate as to the mechanistic basis of the posttreatment effect, which could relate to a characteristic either of *Wolbachia* itself or of the host cells. If the secondary tetracycline binding site in the 30S ribosomal subunit is particularly susceptible in *Wolbachia*, drug concentrations could become insufficient to completely inhibit translation at the primary site during the early posttreatment phase, while maintaining 16S rRNA in an error-prone conformation at the secondary site (6). In this scenario, mistranslated proteins would be synthesized, which is the basis for the bactericidal activity of streptomycin (9), another antibiotic that stabilizes the 30S subunit in the error-prone state (10). Alternatively, accelerated clearance of *Wolbachia* after drug withdrawal could be associated with a greater capacity of host cells to eliminate damaged bacteria from intracellular vacuoles (37), since high concentrations of tetracyclines (10^1 to 10^2 μg/ml) inhibit mitochondrial lipid metabolism, resulting in a toxic effect termed steatosis (2). However, DOX was used at only 0.25 μg/ml in the present study, and no overt toxicity on mosquito cells was evident, although subtle effects on eukaryotic metabolism cannot be excluded.

Notwithstanding the potent posttreatment effect, a minute population of residual, viable *Wolbachia* was still detectable by

16S rRNA RT-PCR after 3 weeks of DOX treatment. These organisms may represent a persistent nonreplicating form (24) with greatly reduced antibiotic susceptibility, as has been reported for other intracellular bacteria, such as *Chlamydia* (13), *Anaplasma* (11), and *Mycobacterium* (17). In addition, this observation challenges the assumption that detection of *Wolbachia* DNA in antibiotic-treated filarial nematodes, frequently observed weeks or months after the initiation of chemotherapy, can be interpreted as amplification from dead organisms (5, 16, 44).

In conclusion, in this in vitro system, we have demonstrated that depletion of viable *Wolbachia* is significantly enhanced after antibiotic withdrawal, relative to the rate of elimination in the continuous presence of the drug. The application of similar methodology to other intracellular bacteria may reveal that this posttreatment effect is of more widespread significance. Moreover, these in vitro observations are entirely consistent with our in vivo findings using *O. ochengi* in cattle (16) and indicate that further investigation is warranted into intermittent, compared with continuous, treatment of *Wolbachia* in filarial nematodes.

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