Evidence against Wolbachia symbiosis in Loa loa

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Abstract

Background: The majority of filarial nematode species are host to Wolbachia bacterial endosymbionts, although a few including Acanthocheilonema viteae, Onchocerca flexuosa and Setaria equina have been shown to be free of infection. Comparisons of species with and without symbionts can provide important information on the role of Wolbachia symbiosis in the biology of the nematode hosts and the contribution of the bacteria to the development of disease. Previous studies by electron microscopy and PCR have failed to detect intracellular bacterial infection in Loa loa. Here we use molecular and immunohistological techniques to confirm this finding.

Methods: We have used a combination of PCR amplification of bacterial genes (16S ribosomal DNA [rDNA], ftsZ and Wolbachia surface protein [WSP]) on samples of L. loa adults, third-stage larvae (L3) and microfilariae (mf) and immunohistology on L. loa adults and mf derived from human volunteers to determine the presence or absence of Wolbachia endosymbionts. Samples used in the PCR analysis included 5 adult female worms, 4 adult male worms, 5 mf samples and 2 samples of L3. The quality and purity of nematode DNA was tested by PCR amplification of nematode 5S rDNA and with diagnostic primers from the target species and used to confirm the absence of Wolbachia endosymbionts. Samples used in the PCR analysis included 5 adult female worms, 4 adult male worms, 5 mf samples and 2 samples of L3. The quality and purity of nematode DNA was tested by PCR amplification of nematode SS rDNA and with diagnostic primers from the target species and used to confirm the absence of contamination from Onchocerca sp., Mansonella perstans, M. streptocerca and Wuchereria bancrofti. Immunohistology was carried out by light and electron microscopy on L. loa adults and mf and sections were probed with rabbit antibodies raised to recombinant Brugia malayi Wolbachia WSP. Samples from nematodes known to be infected with Wolbachia (O. volvulus, O. ochengi, Litomosoides sigmodontis and B. malayi) were used as positive controls and A. viteae as a negative control.

Results: Single PCR analysis using primer sets for the bacterial genes 16S rDNA, ftsZ, and WSP were negative for all DNA samples from L. loa. Positive PCR reactions were obtained from DNA samples derived from species known to be infected with Wolbachia, which confirmed the suitability of the primers and PCR conditions. The quality and purity of nematode DNA samples was verified by PCR amplification of SS rDNA and with nematode diagnostic primers. Additional analysis by
Background
The majority of filarial nematodes are infected with Wolbachia endosymbionts, including the major pathogenic species in humans, Wuchereria bancrofti, Brugia malayi and Onchocerca volvulus [1,2]. Research on the symbiosis of Wolbachia bacteria and filarial nematodes has highlighted the contribution of bacteria to inflammatory disease pathogenesis and the use of antibiotic therapy as a novel method of treatment [3–5]. A few filarial nematode species, including Acanthocheilonema vitaeae, Onchocerca flexuosa and Setaria equina, are free of Wolbachia infection [6–9]. Studies using these species have helped define the contribution of Wolbachia to inflammatory pathogenesis [10–13] and the effects of antibiotic depletion on development and fertility [14,15]. Determining the extent of Wolbachia infection in filarial nematodes could also shed light on the evolutionary history of the symbiosis and give insight into the nature of the mutualistic association.

The association of Wolbachia with severe inflammatory reactions post-treatment of B. malayi and O. volvulus with ivermectin or diethylcarbamazine [10,16,17] prompted us to examine whether L. loa was infected with Wolbachia and thus could potentially contribute to the rare but serious severe adverse neurological events (SAE) following ivermectin treatment [18]. Previous electron microscopy studies have failed to find intracellular bacteria in L. loa microfilariae [6,19] and adults [20,21] and PCR analysis of microfilariae from two patients also failed to detect Wolbachia [22]. Here we have used molecular and immunohistological analysis to confirm this finding in a larger number of samples derived from different endemic areas.

Methods
Parasites
Nematode samples from infected humans and animals were obtained with the approval of the ethics committees and regulatory authorities of all institutions and countries involved in this study.

Loa loa
Microfilariae
Microfilariae samples were obtained from venous blood samples from individuals diagnosed with Loa loa from Cameroon (3), Gabon (2) and Benin (1). Whole blood samples were either frozen directly or filtered to collect microfilariae, which were either frozen, fixed in 80% ethanol or used directly for the extraction of DNA.

Third-stage larvae (L3)
L3 larvae were collected from Chrysops fed on human volunteers from Cameroon. Engorged flies were maintained in insectaries for 12 days at 23–28°C and 77–80% humidity. Heads of infected flies were dissected in RPMI medium and the recovered L3s washed three times. Larvae were either frozen in liquid N2 or used to inoculate a drill, Mandrillus leucophaeus, for the recovery of adult worms.

Adult worms
Two adult female worms were obtained following surgical removal from infected individuals in Gabon and fixed in 80% ethanol. Adult worms (three female and four male worms) were recovered from subcutaneous tissues of a two-year old drill born in captivity, seven months after subcutaneous inoculation with 200 L3 in the inguinal region and fixed with 4% formaldehyde in phosphate buffered saline.

PCR
PCR analyses were conducted in two separate laboratories, in the Liverpool School of Tropical Medicine and the Bernhard Nocht Institute for Tropical Medicine, Hamburg, and are therefore described for each laboratory.

Liverpool
DNA was extracted from the parasites by the phenol/chloroform method, as follows. Worms were placed in 500 µl of TEN (20 mM Tris pH 7.5; 50 mM EDTA; 100 mM NaCl) with 0.5% SDS, 0.1 mg/ml proteinase K and 1 µl β-mercaptoethanol, and incubated in a 55°C water bath until the parasites were digested. Phenol: chloroform: isoamyl alcohol (25:24:1, Sigma, UK) was added to the lysate, gently mixed, and after a 2 minute centrifugation, the aqueous phase was removed to a clean tube. The organic phase was re-extracted with 200 µl TEN and the aqueous phases combined. To precipitate the DNA, 1.2 ml of room temperature ethanol was added and the DNA pelleted by centrifugation, followed by washing with ice cold 70% ethanol, centrifugation, and drying of the pellet; the pellet was then resuspended in 200 µl of sterile distilled water. DNA concentration was determined by absorbance at 260 nm (Adult female, 226, 157 µg/ml; microfilariae 73, 102 µg/ml; L3, 2 µg/ml). By PCR, L. loa samples were confirmed to be positive for L. loa DNA [23] and negative for Wolbachia symbiosis. Immunohistology of L. loa adults and mf confirmed the results of the PCR with no evidence for Wolbachia symbiosis.

Conclusion: DNA analysis and immunohistology provided no evidence for Wolbachia symbiosis in L. loa.
Onchocerca species [24], *M. perstans* and *M. streptocerca* [25] and *Wuchereria bancrofti* [26].

16s rDNA
For amplification of bacterial 16s rDNA, 5 µl of DNA was amplified with the eubacterial primers 27f (5’-GAG AGT TTG ATC CTG GCT CAG-3’) and 1495r (5’-CTA CGG CTA CCT TGT AAC GA-3’) as previously described [10].

ftsZ
To increase the sensitivity of the reaction [27], ftsZ primers (ftsZ UNIF 5’-GG [CT] AA [AG] GGT GC [AG] GCA GAA GA-3’ and ftsZ UNIFR 5’-ATC [AG]AT [AG]CC AGT TGC AAG-3’) [28] were used with a proof-reading DNA primer, 1X buffer, 350 µM dNTPs, 2.5 µM DNA polymerase and between 1.5 mM and 2.5 mM MgCl2. After an initial denaturation at 95°C for 2 minutes, samples were heated at 94°C for 10 seconds, 65°C for 30 seconds, and 68°C for 1.5 mins for a total of ten cycles, after which the samples were amplified for an additional 20 cycles with an annealing temperature of 55°C and an extension time of 68°C for 1.5 mins plus an extra 20 seconds each cycle.

WSP
WSP primers were based on the sequence of *Brugia malayi* Wolbachia WSP (WSP-FILF 5’-CGC TTG CAG TAG AAC AGT GAG-3’ and WSP-FILR 5’-GCT TCT GCA CCA ATA ATT GCA GTA-3’). One microlitre of DNA was amplified with 0.2 µM of each primer, 1X buffer, 350 µM dNTPs, 2.5 µM DNA polymerase and between 1.5 mM and 2.5 mM MgCl2. After an initial denaturation at 95°C for 2 minutes, samples were heated at 94°C for 10 seconds, 65°C for 30 seconds, and 68°C for 1.5 mins for a total of ten cycles, after which the samples were amplified for an additional 20 cycles with an annealing temperature of 55°C and an extension time of 68°C for 1.5 mins plus an extra 20 seconds each cycle.

PCR products were visualised on an agarose gel stained with ethidium bromide.

Hamburg
Individual *L. loa* worms (4 male, 3 female) or microfilariae were homogenised in lysis buffer (50 mM Tris-HCl, pH 8; 20 mM EDTA; 2% SDS), then incubated for 30 minutes at 37°C with 0.1 volume of 10 mg/ml Proteinase K (Qiagen, Hilden, Germany). The DNA was extracted twice in phenol:chloroform, ethanol precipitated, and the pellet was resuspended in 200 µl water. The DNA concentration as determined by absorbance at 260 nm had a range of 15–145 µg/ml with an average of 53 µg/ml. PCR of the nematode 5S rDNA was performed as previously described [25] to confirm the quality of the DNA.

The following primer sets and annealing temperatures were used to amplify the eubacterial 16S rDNA and ftsZ sequences: 16S rDNA forward: AGA GGT TGA TCC TGG CTC AG, reverse: AAG AGG TGA TCC AGC C [14]; ftsZ forward: CIT GGT GCT GGT CCT TG GCC, reverse: TAA GTA TTG CTG AAC ATC CAA. PCR was performed on 2 µl of genomic DNA in a 50 µl reaction in 1X Hotstar Taq® buffer (Qiagen, Hilden, Germany) with 1.5 mM MgCl2, 0.2 µM dNTPs, and 20 µM of each primer. The cycle conditions were an initial step of 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 2 minutes, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Products were separated on agarose gels in 1X TBE and visualised with ethidium bromide. FtsZ primers were also used with the Elongase® taq polymerase mix (Invitrogen, Paisley, United Kingdom) with 2 mM Mg2+ as per the manufacturer’s protocol.

**Immunohistology**
**Antiser to recombinant Brugia malayi Wolbachia WSP**
A rabbit was immunised and boosted with purified recombinant *Brugia malayi* Wolbachia WSP protein and the serum tested in a Western blot. A single band of 28 kDa was detected in *B. malayi* protein extract, whereas there was no recognition of a Wolbachia-free *A. viteae* extract or when pre-immunisation serum was used (not shown). Likewise, when used in immunohistology, this antibody specifically labelled *Wolbachia* from 14 species of filarial nematodes tested but did not cross react with any nematode tissue (D. W. Büttner, pers. comm.; our unpublished observation).

**Immunoelectron microscopy**
*L. loa* microfilariae were fixed and embedded for immunoelectron microscopy as described previously [29]. Sections cut at 90 nm and mounted on nickle grids were blocked with 1% bovine serum albumin in PBS with 0.01% Tween 20 and then reacted with rabbit anti-WSP serum (dilutions of 1 in 20 to 1 in 100), washed and incubated with goat anti-rabbit colloidal gold conjugate (20 nm diameter, British Biocell, UK). Sections were counterstained with 2% aqueous uranyl acetate solution and examined on a Phillips CM10 transmission electron microscope.

**Light immunohistology**
*L. loa* adult worms fixed with 4% formaldehyde in phosphate buffered saline were embedded in paraffin. Sections were probed with rabbit anti-WSP serum (1:250) and visualised using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method according to the manufacturer’s recommendations (Dako Diagnostika, Hamburg, Germany). Anti-rabbit mouse immunoglobulin was used as a secondary antibody (clone MR12/53, Dako Diagnostika) and Fast Red TR salt (Sigma) as the chromogen with
haematoxylin (Merck) as the counterstain. *Brugia malayi* adult female worms were used as a positive control.

**Results**

**PCR**

To determine the presence of *Wolbachia* in *L. loa* at the molecular level, PCR was performed on genomic DNA with primers for the eubacterial 16S rDNA, *ftsZ* and WSP sequences. No PCR product was obtained with any of the primer sets with *L. loa* and *A. viteae* DNA (Figure 1), although all DNA samples produced a nematode 5S rDNA signal, indicating that there was DNA at sufficient concentration for detection in one round of PCR. The 16S rDNA, *ftsZ* and WSP primers were functional as all primer sets produced a visible product at the expected molecular weight in the positive controls (Figure 1). Additional analysis by 'long PCR', which has been reported to increase the sensitivity of the identification of *Wolbachia* in arthropods [27], was used; however, neither the Elongase® polymerase mix nor the Bio-X-Act polymerase used with the *ftsZ* primer sets produced a signal from *L. loa* of the expected molecular weight (Figure 1).

**Immunohistology**

No labelling of WSP was detected in sections of *L. loa* microfilariae by immuno-electron microscopy. Light immunohistology of *L. loa* adult worms showed no labelling of male or female worms (Figure 2). Labelling of positive controls (*B. malayi*) confirmed the specificity of the antisera to *Wolbachia* (Figure 2).
Figure 2
Light immunohistology of *Loa loa* adult worms with antisera against *Wolbachia* surface protein. (A-D) Adult female *L. loa* showing lack of staining in lateral cord (LC), oocytes (O) and morula (M) stages (magnification × 160). (E) Adult male showing lack of staining in lateral and median cords (LC, MC) and testis (T) (magnification × 100). (F) Adult female *B. malayi* showing positive staining of *Wolbachia* in the lateral cords (LC) and oocytes (O) (magnification × 160).
Discussion

Here we present data of molecular and immunohistological analyses that failed to provide evidence for Wolbachia symbiosis in *L. loa*. This confirms previous findings on *L. loa* by electron microscopy and PCR [6,19–22] and extends these observations to a larger sample of adult worms, infective larvae and isolates of microfilariae from three different endemic areas.

The release of Wolbachia into the blood following antifilarial chemotherapy has been shown to be associated with severe systemic inflammatory reactions in individuals infected with *O. volvulus* or *B. malayi* [16,17]. One of the objectives of this study was to determine whether Wolbachia might contribute to the rare but severe neurological adverse events following ivermectin treatment of *L. loa* [18]. We conclude that the neurological consequences of SAE following ivermectin treatment of individuals with *L. loa* are not associated with Wolbachia. In people co-infected with *L. loa* and *O. volvulus* or *W. bancrofti*, adverse events induced by Wolbachia derived from the latter two species may nevertheless contribute to post-treatment reactions. Double blind placebo-controlled trials to evaluate the effect of doxycycline depletion of Wolbachia on the development of post-treatment reactions to filarial chemotherapy are currently underway in individuals infected with *B. malayi*, *W. bancrofti*, *O. volvulus*, and co-infection with *O. volvulus* and *L. loa*. In two patients with *L. loa* treated with doxycycline for six weeks (200 mg/day), microfilaraemia was still detected at 120 days of follow up [22].

Studies on species of filarial nematodes infected with Wolbachia suggest that the symbiosis exists throughout all samples of populations and individual parasites [1,2]. The ubiquity of infection, congruence with host phylogeny and deleterious effects of antibiotic clearance on embryogenesis, development and viability suggest a mutualistic dependency [1,2]. It is clear, however, that some species of filariae, including *L. loa*, can cause widespread infection without the need for bacterial symbionts. Although further studies are needed it has been suggested that the absence of Wolbachia in *A. viteae* and *Setaria* sp. is a consequence of their divergence from the lineage prior to the acquisition of Wolbachia infection. Conversely, the absence of Wolbachia from *O. flexuosa* and *L. loa* is more likely to be due to the loss of bacterial symbionts [2]. Further analysis incorporating the results of the present study could provide additional insights into the evolutionary biology of the filarial nematode-Wolbachia symbiosis.

Although data collected so far support the conclusion that filarial nematode species with evidence of symbiosis are ubiquitously infected, sampling of these species is inevitably limited and we cannot rule out the possibility that populations or individual nematodes exist without infection. Similarly with species shown to be aposymbiotic, populations may exist that contain symbionts, particularly if the absence of bacteria is due to a secondary loss of Wolbachia. In this regard it would be worthwhile to analyse samples of monkey strains of *L. loa*, which may be ancestrally ‘primitive’ compared to the strain parasitising humans. Additional studies on the extent of Wolbachia symbiosis in infected species and the infection status of the human filariae *M. perstans* and *M. streptocera* are important areas for future research.

Conclusions

We conclude that this study provides no evidence for Wolbachia symbiosis in *L. loa*. It is therefore highly improbable that Wolbachia contributes to the neurological consequences of SAE following ivermectin treatment in individuals with infections of *L. loa* unaccompanied by other filarial species.

Competing interests

None.

Authors’ contributions

Helen McGarry – PCR analysis, preparation of draft manuscript

Ken Pfarr – PCR analysis, preparation of draft manuscript

Gill Egerton – immunohistology

Achim Hoerauf – Interpretation of PCR data

Jean-Paul Akue – Collection, identification and processing of *L. loa*

Peter Enyong – Collection, identification and processing of *L. loa*

Samuel Wanji – Collection, identification and processing of *L. loa*

Sabine Kläger – Collection, identification and processing of *L. loa*

Ted Bianco – Collection, identification and processing of *L. loa*

Nick Beeching – Collection, identification and processing of *L. loa*

Mark Taylor – Interpretation of data and preparation of final manuscript
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