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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 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 5S rDNA and with nematode diagnostic primers. Additional analysis by

'long PCR' failed to produce any further evidence 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*.

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 viteae, 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 immunohistochemical 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 N_2 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

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 TAC 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 polymerase enzyme (Bio-X-Act, Bioline, U.K.). One microlitre of DNA was amplified with 0.4 μM of each primer, 1 X buffer, 350 μM dNTPs, 2.5 U DNA polymerase and between 1.5 mM and 2.5 mM $MgCl_2$. 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 TAC AAT AGT GAG-3' and WSP-FILR 5'-GCT TCT GCA CCA ATA GTG CT-3'). One microlitre of adult or 5 μl of microfilarial/L3 DNA was amplified with 0.2 μM of each primer, 1X buffer that contained 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 2.5 U of HotStarTaq DNA polymerase and water to 50 μl. Following activation of the DNA polymerase at 95°C for 15 minutes, the mixes were heated at 94°C for 45 seconds, 60°C for 45 seconds with a decrease of 1°C per cycle for 5 cycles, then at 55°C for 35 cycles, with an extension step at 72°C for 90 seconds and a final extension step of 8 minutes.

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 GTT TGA TCC TGG CTC AG, reverse: AAG AGG TGA TCC AGC C [14]; ftsZ forward: CTT GGT GCT GGT GCT TTG CCT, reverse: TAC CAA TCA TTG CTT TAC CCA. 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 $MgCl_2$, 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 Mg^{2+} as per the manufacturer's protocol.

Immunohistology

Antisera 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).

Immuno-electron 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

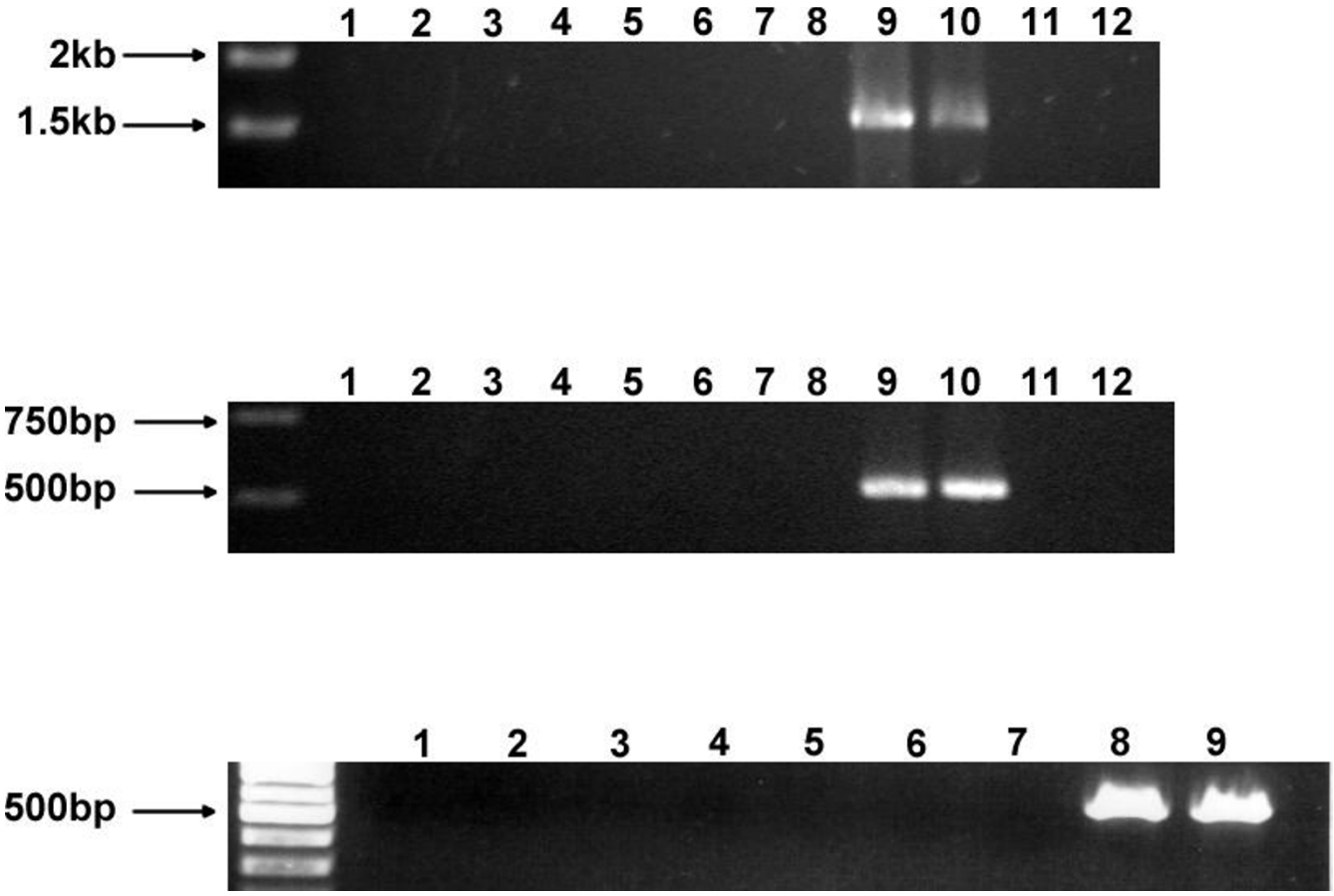

Figure 1
Detection of *Wolbachia* in *Loa loa* by PCR. A) Primers for eubacterial 16S rDNA only amplify a signal in *Onchocerca volvulus* positive controls (lanes 9 and 10). B) Primers for *Wolbachia* ftsZ only amplify DNA from *O. volvulus*. Lane description for A+B: 1–3: *L. loa* female worms, lanes 4–7: *L. loa* male worms, lane 8: *L. loa* microfilariae, lanes 9–10: *O. volvulus*, lanes 11–12: *Acanthocheilonema viteae*. C) Elongase® Taq, having 3'–5' proofreading activity, only detects endosymbiont DNA in the *O. volvulus* controls. Lanes 1–3: *L. loa* female worms, lanes 4–7:*L. loa* male worms, lanes 8–9: *O. volvulus*.

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).

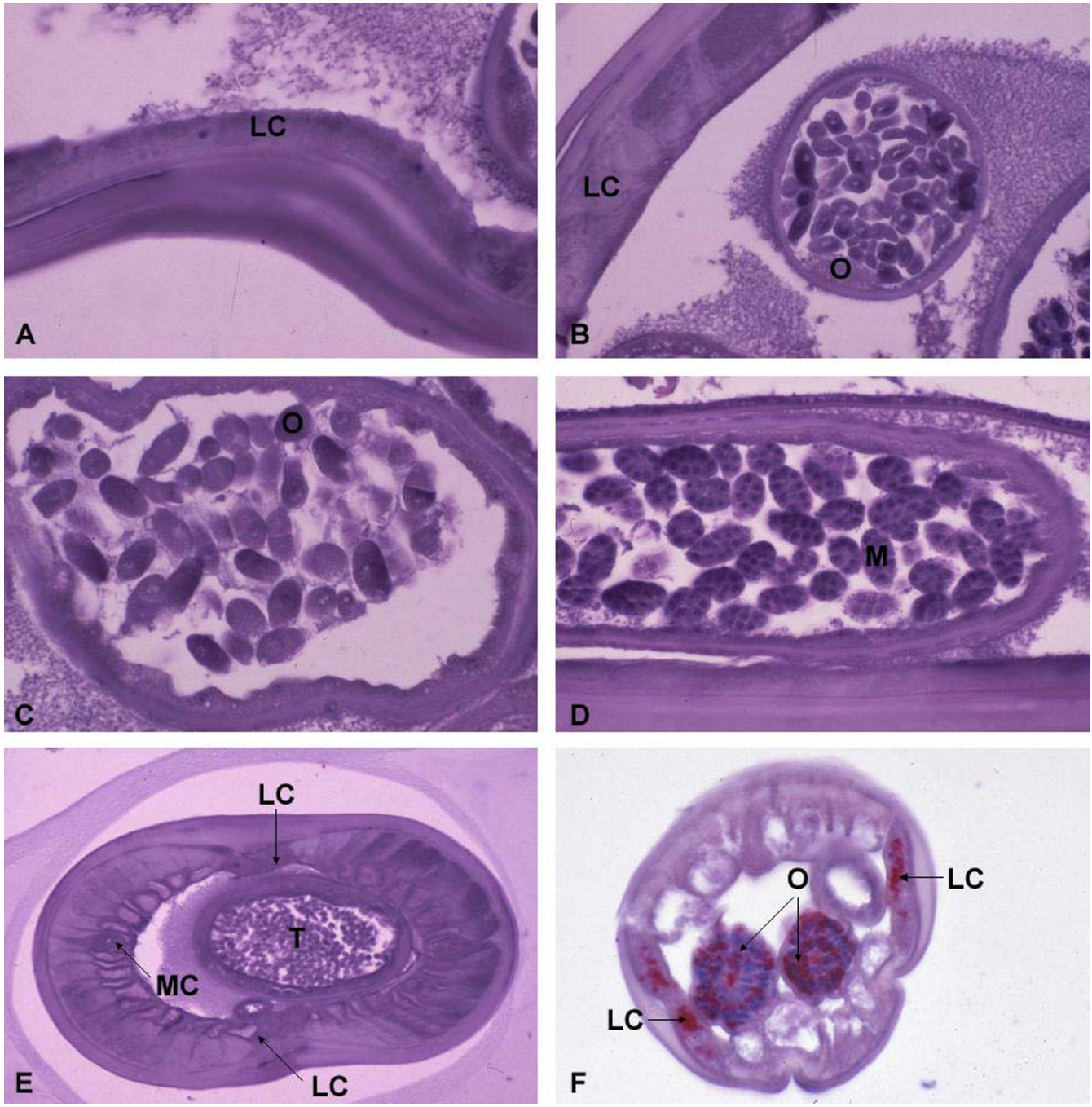

Figure 2
Light immunohistology of *Loa loa* adult worms with antisera against *Wolbachia* surface protein. (A-D) Adult female *L. loa* showing lack of staining in lateral cord (LC), oocytes (O) and morula (M) stages (magnification × 160). (E) Adult male showing lack of staining in lateral and median 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 coinfected 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 coinfection 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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