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Wolbachia-Filarial Nematode Interactions: Embryological, Cellular and Molecular Aspects and Therapeutic Targets

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Review Article

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Abstract

Filarial nematodes including *Wuchereria bancrofti, Onchocerca volvulous, Brugia malayi, B. timori* and *Loa loa* cause some of the most common parasitic infections in humans including elephantiasis, blindness and skin lesions. *Wolbachia*, an *α-proteobacterium* is the most widespread endosymbiotic bacterium widely present in various insect species, in parasitic nematodes of animals and also in the plant-parasite nematode, *Radopholus similis*. In insects, they harbour in male and female germlines and in a variety of somatic tissues. In filarial nematodes, they live as reproductive endosymbionts in ovarian tissues, oocytes and oogonia and newly forming embryos within the uterus and affect moulting, reproduction and survival of the host nematode. They provide essential metabolites such as riboflavin, haem and nucleotides to the host nematodes, but they cannot synthesize some vitamins, coenzyme A, NAD, biotin, *folate*, ubiquinone, *lipoic* acid etc. de novo due to incomplete biochemical pathways, and derive those from host nematodes. Thus, there is an obligate symbiotic relationship between *Wolbachia* and host nematode. Antibiotic treatment against *Wolbachia* has been proved as a promising target for prevention of *filariasis* which can break the parasitic cycle. Genomic studies regarding embryological, cellular and molecular aspects of *Wolbachia* and filarial nematode are still limited. Further research in this area could employ *Wolbachia*-filarial nematode interactions as a potential therapeutic target to reduce or prevent filarial infections.

Keywords: *Wolbachia*; Filarial Nematode; *Brugia Malayi*; Blastomere; Aldolase

Abbreviations: LF: Lymphatic Filariasis; GPELF: Global Programme to Eliminate Lymphatic Filariasis; AB: Anterior Blastomere; PI: Posterior Blastomere; WSPs: *Wolbachia* Surface Proteins; LGT: Lateral Gene Transfer.

Introduction

Tissue-dwelling filarial nematodes including *Wuchereria bancrofti, Onchocerca volvulous, Brugia malayi, B. timori and Loa loa* are the causative agents of some of the most common parasitic infections in humans including lymphatic filariasis commonly known as elephantiasis, blindness and extensive skin lesions [1,2]. The causative agents of lymphatic filariasis (LF), *Wuchereria bancrofti, Brugia malayi and B. tumori* (the first one is most abundant) affect approximately 38.5 million individuals worldwide and another 950 million individuals have the risk to be infected in 54 countries [3]. The target set by the Global Programme to Eliminate Lymphatic Filariasis (GPELF) in 2000 to eliminate LF as a public health problem globally by 2020 was not achieved by then. Another target

has been set to achieve this goal by 2030 [4].

Wolbachia, an α-proteobacterium is the most widespread endosymbiotic bacterium widely present in arthropod species, including a large proportion of insects, i.e., fruitflies, mosquitoes, tsetse flies, bugs, ants and termites [5-17]. They are also present in animal parasitic nematodes and in the plant-parasitic nematode *Radopholus similis* [18-21]. Lateral gene transfer of *Wolbachia* DNA into the *Wolbachia*-infected nematode genome has been reported [2,22]. *Wolbachia* lives symbiotically with the filarial nematodes of the two subfamilies, the Onchocercinae and the Dirofilariinae and affects molting, reproduction and survival of the host nematode [17,23]. They are pleiomorphic organisms varying from 0.2 to 4 micometer in size. They reside in the vacuoles derived within the host and form obligate intracellular niche [24]. They also live in ovarian tissues, oocytes and oogonia and newly forming embryos within the uterus of the nematode [25]. Each vacuole can harbor single bacterium. The vacuole is expanded along the bacteria during division to form two distinct vacuoles. But when growth is rapid, a single vacuole can contain clusters of bacteria nematode [26]. There is an obligate mutualistic symbiotic relationship between *Wolbachia* and pathogenic filarial nematodes including W. *bancrofti, B. malayi, B. timori, Oncocerca spp., Dirofilaria spp. and Litomosoides sigmodontis* [19,20,25]. But the basis for this relationship is not established yet. Previous studies suggest that *Wolbachia* may provide essential metabolites such as riboflavin, haem and nucleotides. Conversely, genomic studies showed that the host nematode provides metabolites to the *Wolbachia*. This endosymbiont cannot perform de novo synthesis of some vitamins, coenzyme A, NAD, biotin, folate, ubiquinone, lipoic acid etc. due to incomplete biochemical pathways. However, recent genomic analysis of both *Wolbachia* and its nematode host provides clues for their cellular and molecular aspects of interactions. In this literature study will address the role of *Wolbachia* in parasitism by filarial nematodes.

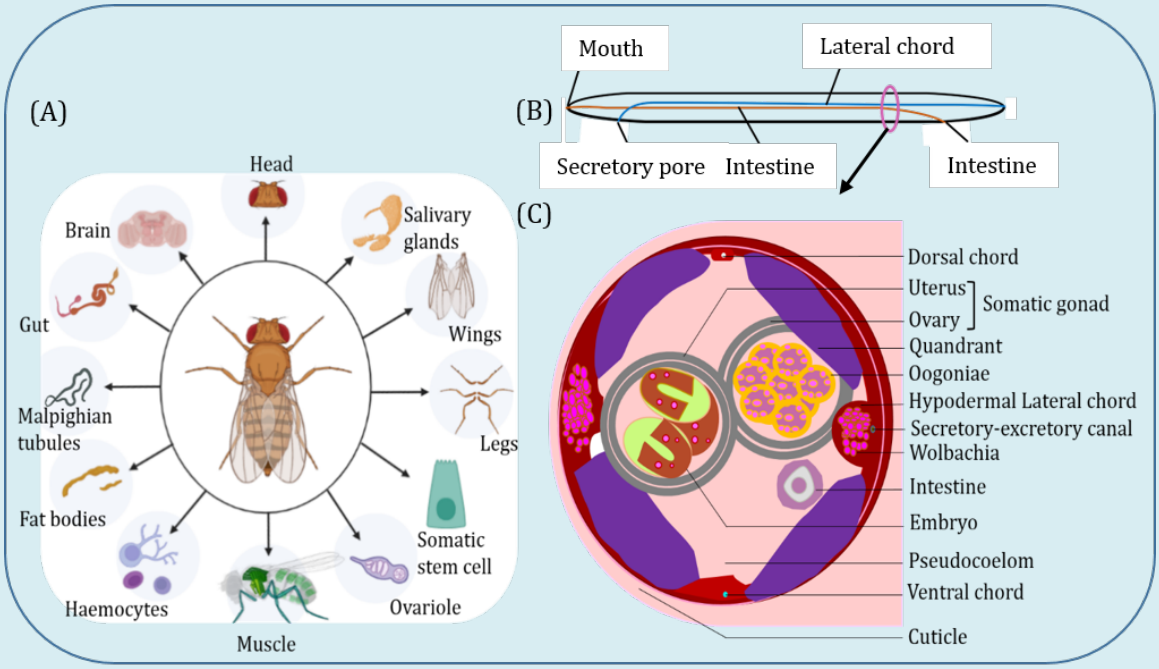

Figure 1: Diagrams showing localization of *Wolbachia* in insects and filarial nematodes. (A) Presence of *Wolbachia* as endosymbionts in different organs of insects (adapted from [5-9,13-17]). (B) Simplified structure of an adult female filarial nematode. (C) Schematic representation showing the localization of *Wolbachia* in a cross section of adult female filarial nematode, *B. malayi*. *Wolbachia* have been reported in the hypodermal lateral chords, germline (oogoniae), secretory-excretory canal and embryos (adapted from [25,27,28]).

Mechanism of Segregation of *Wolbachia* in Filarial Nematodes

The transmission of *Wolbachia* bacteria in the filarial nematode, *B. malayi* from embryonic to adult stages has been explored with the development of whole-mount immunofluore scence technique. By using this technique, the bacteria has been tracked at cellular and tissue levels in the different developmental stages of host nematode i.e., during fertilization, initial zygotic divisions, embryonic divisions and adult worms. During fertilization, *Wolbachia* are localized ubiquitously in the oocyte with highest abundance at the

anterior end of the egg. After fertilization, at the migration step of pronuclei, their distribution changes, they remain restricted at the posterior end of the egg. Subsequently, they begin segregating following zygotic divisions. When the larger anterior blastomere (AB) and smaller posterior blastomere (PI) are formed by the early P0 zygotic division, most of the *Wolbachia* localize in PI by asymmetric segregation. AB produces ABa and ABb by symmetric cell division. The amount of *Wolbachia* in these descendants is variable, however, always poorer than in the direct descendants of the P1 lineage. Later, when the PI divides to form anterior somatic blastomere EMS and posterior germ line blastomere P2, they favorably become segregated in the P2. The EMS, following three successive divisions divides to form MS (ectodermal and mesodermal derivatives, pharynx and somatic gonad) and E (endoderm or gut). The division of P2 produces a dorsally localized C blastomere and a posteriorly located P3 blastomere. *Wolbachia* mostly segregate to the C blastomere and a small number segregate to the P3 blastomere. Subsequent divisions of C blastomere produce muscle cells and hypodermal cells. The hypodermal cells have three regions namely dorsal, lateral and ventral hypodermal chords. During morphogenesis, *Wolbachia* localize in dorsal posterior hypodermal cells, but they are absent from almost all anterior cells including pharyngeal cells, neuroblasts, gut and muscle cells [25].

The transmission of *Wolbachia* into the hypodermal chords varies not only between nematodes but also within an individual nematode. In some cases, only one of the chords contains *Wolbachia*, whereas in others 50% of each chord is known to be infected. It has been found that when fusion of individual hypodermal chord cells occur to form syncytium, *Wolbachia* spreads throughout the hypodermal chord. Division of P3 produces muscle cells and P4. P4, the germline precursor divides symmetrically to from two primordial germ cells, Z2 and Z3. *Wolbachia* that localize in P3 blatomeres segregate towards half of the P4 blastomeres or Z2 and Z3 germline cells, while the rest half blastomeres remain non-infected. The embryos with *Wolbachia* infected blastomeres become females and those with *Wolbachia* non-infected become males. This cell-linage specific form of transmission of *Wolbachia* from P3 blastomere to Z2 and Z3 germ cells, thereby to the next generation of nematodes indicates that *Wolbachia* are vertically transmitted from host mother to offspring [25]. To sum up, presence of *Wolbachia* in the C blastomere is the main source of transmission to the hypodermis, and at the same time gradual maintenance via P2, P3 and P4 ensures their transmission to the germline (Figure 2).

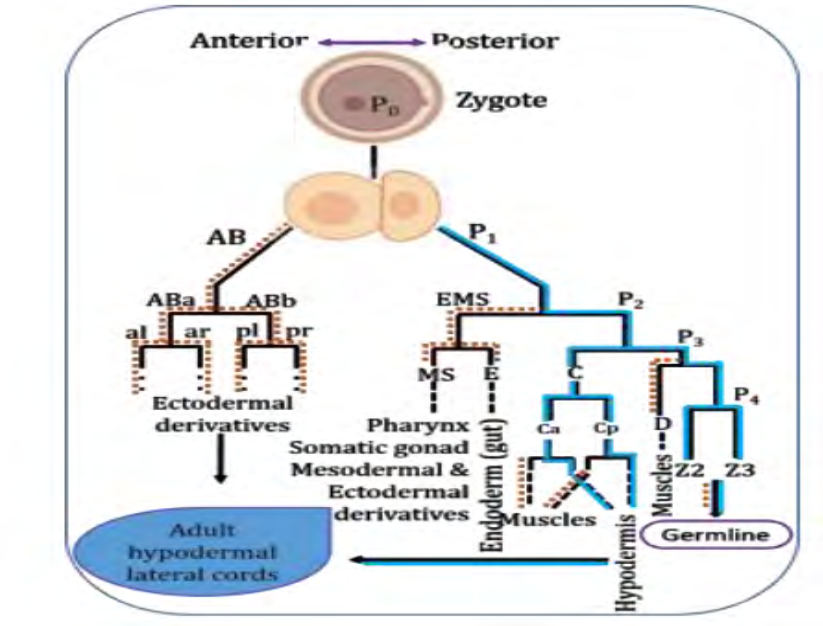

Figure 2: Mode of segregation of *Wolbachia* in filarial nematode at cellular level during early embryogenesis. Blue solid lines indicate the route of transmission of *Wolbachia* towards the germline precursors in the females and orange dots indicate the C lineage leading to hypodermal cells (adapted, with permission, from [25]).

A recent study investigated distribution of *Wolbachia* during the different stages of life cycle of *B. malayi*. Results showed that *Wolbachia* were absent from reproductive tissues of both sexes from at least 3rd larval instar to young adults [29]. In the young adult females, all the *Wolbachia* are present in the lateral hypodermal chords neighbouring

ovaries or testis, but not present in in the germinative zones in the ovaries. Following 3 weeks after the moulting of 4th and 5th larval instars, the germinative zones become enriched with *Wolbachia*, which indicates that *Wolbachia* invade from the nearby hypodermal chords. The pattern of this kind of cell-to- cell invasion was confirmed in another study with four species of Onchocercidae, B [30]. Malayi and Litomosoides sigmondontis, Dirofilaria immitis and Onchocerca dewittei japonica. The presence of *Wolbachia* like sequences has also been reported in the reproductive tissues of a plant-parasitic nematode, Radophoilus similis but the role of this bacteria and its mode of transmission is yet to be explored. The localization of *Wolbachia* in the nematode host tissue at cellular level is very important to understand the *Wolbachia*-filarial nematode interactions because administration of anti-*Wolbachia* drugs then would be possible to target specific nematode cells.

How Do Filarial Nematodes Suffer and/or Benefit From the Endoparasite *Wolbachia*?

Synthesis of Essential Metabolites by *Wolbachia*

The discovery of complete genome sequence and analysis of *B. malayi* host and its endosymbiont, *Wolbachia* made it possible to compare the biosythetic capabilities between them [31]. Draft genome sequence of filarial nematode *B. malayi* reveals that the nematode lacks most of the enzymes that are required for biosynthesis of essential metabolites: 9 out of 10 enzymes essential for de novo purine synthesis, all the 5 enzymes essential for riboflavin synthesis and 6 out of 7 genes essential for heme synthesis [31]. Hence, the nematode must depend on its host or endosymbiont to fulfil the requirement of these essential metabolites [1]. *Wolbachia* synthesizes nucleotides not only to fulfil its own demand, but also supplement the nucleotides of the host nematode when needed, for instance during oogenesis and embryogene when the host has the requirement for high amount of DNA. On the other hand, *Wolbachia* lacks the machineries for de novo biosythesis of vitamins and co-factors including biotin, Coenzyme A, NAD, ubiquinone, lipoic acid, pyridoxal phosphate and folate. *Wolbachia* gets these precursors from the respective host nematode [1].

Although a number of genomes of *Wolbachia* have already been sequenced, knowledge regarding the relationship of this endosymbiont with its hosts is still poor. In a recent study [32], sequencing of the genome and transcriptome analysis of *Wolbachia* from filarial nematode Onchocera ochengi (wOo) showed some differences between wOo and wBm (*Wolbachia* from *B. malayi*) in relation to nutritional provisioning, insertion sequence elements and a variety of proteins that comprise repeat motifs. Unlike the wBm, most of the enzymes responsible for the synthesis of riboflavin have been lost or pseudogenized in wOo except only one gene ribA remaining intact. Moreover, "the incomplete pathways for biotin, pantothenate, and coenzyme-A biosynthesis in wBm have been almost entirely lost from the wOo genome, and isoprenoid synthesis (secondary metabolism) is also severely restricted". Two major metabolism-related subunits have been identified in the wOo proteome, one is "succinyl-CoA synthetase" and the other is "ATP synthase". These two enzymes produce purine nucleoside triphosphates from TCA cycle and the respiratory cycle, respectively. Succinyl-CoA synthetase is also an essential precursor for the heme biogenesis. The involvement of wOo in the TCA cycle and the respiratory cycle, and generation of triphosphates (ATP) in somatic tissue of Onchocera ochengi proves that *Wolbachia* has an important function like mitochondria.

Wolbachia surface proteins (WSPs) are known to interact with the host *B. malayi* glycolytic enzymes i.e., aldolase and enolase and also with cytoskeleton i.e., actin to maintain endosymbiosis [23]. Recently, two types of surface proteins namely wBm0152 and wBm0432 have been identified in excretory/secretory products of adult female *B. malayi*. These proteins have specific binding ability with crude protein extracts of *B. malayi* and individual filarial proteins to form functional complexes. The wBm0432 is known to interact with *B. malayi* glycolytic enzymes such as aldolase and enolase, whereas wBm0152 is known to interact with the cytoskeletal proteins of the host including actin and tubulin. It has been found that wBm0152 and host actin as well as wBm0432 and host aldolase co-localize in the vacuole that surround *Wolbachia*. In vitro overlay assay uncovered the interaction of two *Wolbachia* WSPs with the *B. malayi* binding proteins. The assay showed a strong binding of wBm0432 with aldolase and enolase. To confirm if the wBm0432 binds specifically to the *B. malayi* host aldolase, the overlay assay was again done with 'recombinant *B. malayi* His-tagged aldolase', which confirmed the binding of HiswBm0432 with His-Bm-aldolase. Moreover, ELISA-based interaction assay showed strong binding of wBm0152 with bovine actin, which has more than 90% similarity with *B. malayi* actin. Thus, wBm0432 has specific interaction with *B. malayi* aldolase and enolase, and wBm0152 has specific interaction with *B. malayi* cytoskeleton [24].

Effect on Biology and Survivability of Filarial Nematodes

B. malayi and *Wolbachia* have such a mutualistic symbiotic relationship that neither of the partners can survive without the other. Treatment of rodents infected with the filarial worm, L. sigmodontis with tetracycline results in clearance of *Wolbachia* from the worm, and block or delay larval development producing shorter larvae [22]. Administration of another drug, Corallopyronin at 35 mg/

kg/day for 28 days also produce significantly shorter worms (9.0 mm) than the control worms (38 mm) [33]. Another study reported that depletion of *Wolbachia* from *B. malayi* causes an extensive apoptosis of adult germline cells, somatic cells and embryos of microfilariae [34]. In a recent study administering doxycycline in gerbils infected with *B. malayi* revealed considerable changes in expression for 546 *B. malayi* and 200 *Wolbachia* array elements. Results showed upregulation of most (98.5%) diffrentially expressed genes in *Wolbachia*, whereas dowregulation of many more differentially expressed *B. malayi* genes (85%). Genes for reproduction including gender-regulated genes, amino acid metabolism collagen, cytoskeleton and ribosomal processes were down-regulated in response to doxycycline treatment. On the other hand, genes for survival in response to stress including nutrient transport, energy metabolism, anti-oxidants, electron transport, immune evasion, and bacterial signaling pathways were upregulated. These results indicate that *Wolbachia*-infected filarial nematodes are to some extent able to compensate the loss of *Wolbachia* with a view to survive, although they loss reproductive capacity.

Expression analysis of one nematode gene (gst) and two *Wolbachia* genes (tsZ and wsp) were carried out at different developmental phases of *B. malayi* [35]. Each gene copy represented one *Wolbachia*. The bacteria/worm ratio was lowest number in microfilariae, L2 and L3 (larval stages in mosquito) stages. Within a week of infection of host mammal, the bacteria/worm ratio increased 10 times than the prior stages and continued throughout L4 development. In mature female worms, when the ovary and embyonic stages of larvae became infected, the number of bacteria increased. But in adult male worms, the bacterial population was constant. It is evident from the results that *Wolbachia* is associated with development of *B. malayi* larvae in mammalian host as well as long-time survival of the adult worms.

Lateral Gene Transfer from *Wolbachia* to its Host

In eukaryotic organisms, lateral gene transfer (LAT) is an uncommon occurrence, since the germline in eukaryotes is segregated from the other tissues. However, extensive lateral gene transfer (LGT) from *Wolbachia* DNA into the genome of *Wolbachia*-infected filarae has been reported in some studies [2,22]. Whole genome sequencing of *Wolbachia*-depleted *B. malayi* revealed the extent of LGT from *Wolbachia* to its host. Results showed that a significant portion (at least 10.6% or 115.4 kbp) of the 1.08 Mbp wBm genome has been transferred to its *B. malayi* host. The genes showed high similarity to wBm genome including 227 wBm genes and also gene fragments. Among those genes, 32 contain complete open reading frames having the ability to synthesize functional proteins. Furthermore, 4 have the ability of regulating life cycle stage-related transcription. These showed similarity with transcripts from other nematodes meaning that they are functional [22]. *Wolbachia* gene fragments have also been reported in nuclear genome of two distantly related *Wolbachia*- free filarial nematodes, *Acanthocheilonema viteae* and Onchocerca *flexuosa*. Screening using 454 pyrosequencing in these two species identified 114 *Wolbachia*-like DNA sequences in *O. flexuosa* and 49 in *A. Viteae*. Gene expression using qRT-PCR reactions identified 56 *Wolbachia*-like sequences in *O. flexuosa* and 30 in *A. Viteae*. About 50% of these genes are transcribed from pseudogenes. In situ hybridization confirmed that two of these pseudogene transcripts were exclusively expressed in testes and developing embryos of both species. It is evident from these results that, *Wolbachia* was present in the last common progenitor of *Acanthocheilonema viteae* and Onchocerca *flexuosa*, indicating that *Wolbachia* contributed to the genome evolution [2].

Wolbachia-Filarial Nematode Interactions as a Therapeutic Target

Comparative genomic analysis of *Wolbachia*-free or *Wolbachia* associated filarial nematode, and *Wolbachia*-associated nematode species before and after *Wolbachia* clearance has uncovered the molecular and metabolic basis of symbiotic interactions between *Wolbachia* and their host nematodes. The growing genomic knowledge provides new clues for discovery of *Wolbachia* based drug target. A number of studies showed that administration of drugs of tetracycline group such as doxycycline and rifampicin changes the gene expression in response to *Wolbachia* depletion in *Wolbachia*-nematode system [33,36,37]. Anti-*Wolbachia* treatment with tetracycline shows transcript changes in *B. malayi* [36]. After both one- and two weeks of treatment, it was observed that transcripts for synthesis of amino acids and translation of protein were upregulated. This indicates a generalized stress response induced in the nematode because of shortage of essential metabolites that the nematode would derive from the *Wolbachia*. On the other hand, transcripts involved in cuticle biosysthesis were downregulated which reflects an interruption in the normal embryonic programme. When the *Wolbachia* bacteria were exposed to tetracycline in culture, significant amount of endobacteria were died by day 5. Corallopyronin A (Cor A) is another promising drug effective for *Wolbachia* depletion which act as a non-competitive inhibitor of RNA polymerase of *Wolbachia*.

Administration of Cor A in mice infected with *L. sigmodontis* blocks RNA transcription in *Wolbachia* resulting its death, and thus *Wolbachia* clearance occurs in the host nematode *L. sigmodontis*. Mice treated with this drug produces shorter worms (9 mm) compared to control (38 mm) [33]. An intracellular defense strategy called autophagy

is as a major immune system regulator in *Wolbachia* that maintain its population size inside the host nematode, *B. malayi*. Autophagy regulation has been reported as an effective strategy to control *Wolbachia* population in the host nematode [24]. Administration of an autophagy activator, rapamycin in microfilaria and L3- and L4 larvae of *B. malayi* for 5 days resulted in reduced population size of *Wolbachia* in all these developmental stages. When adult female worms were treated with this drug for 7 days, *Wolbachia* population was reduced the bacterial population more than two times. siRNA silencing of target of rapamycin in *B. malayi* (bmTOR) in adult female worms resulted significant reduction of *Wolbachia* population size compared with siGFP treated controls after 7 days of treatment. Moreover, siRNA silencing of ATGI, that initiates autophagy inhibits autophagy, thereby resulted in increased *Wolbachia* population size in adult worms [24]. Therefore, suppression and activation of autophagy by pharmacological or genetic strategies can regulate *Wolbachia* population in *B. malayi*.

Conclusion and Recommendations

Wolbachia play an important obligatory role at cellular and molecular level in the filarial nematodes. The bacteria provide essential metabolites to the host nematode. In addition, *Wolbachia* plays an important role in reproduction and survival of the host nematode. On the other hand, *Wolbachia* gets vitamins and co-factors including biotin, Coenzyme A, NAD, ubiquinone, lipoic acid, pyridoxal phosphate and folate from the host nematode. Thus, there is an obligate symbiotic relationship between *Wolbachia* and host nematode. Antibiotic treatment against *Wolbachia* has been proved as a promising target for prevention of filariasis, which can break the parasitic cycle i.e., reducing and/or inhibiting production of microfilaria in the host nematode. Genomic studies regarding cellular and molecular aspects of *Wolbachia* and filarial nematode are still limited. More research in this area could discover novel solutions to control filarial nematodes.

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