Use of in situ Hybridization to Localize Wolbachia during Embryogenesis in Brugia malayi

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Abstract

Wolbachia are intracellular bacteria that are required for development and reproduction in most filarial nematodes. New approaches are needed to improve understanding of the role of Wolbachia in filarial biology. Recent studies using fluorescent immunohistochemistry revealed asymmetric segregation of Wolbachia in early embryos of Brugia malayi, and this helps to explain the restricted distribution of Wolbachia in adult worms. However, the distribution of Wolbachia in late stages of embryo development or in infective larvae (L3) is largely unknown. In this study we have explored the use of in situ hybridization (ISH) to localize Wolbachia and Wolbachia gene expression during embryogenesis and morphogenesis in B. malayi.

ISH with a 16S rRNA probe revealed Wolbachia in the lateral cords, oocytes, and embryos of B. malayi female worms. This was consistent with prior studies that detected Wolbachia by immunohistochemistry. ISH with probes for Wolbachia surface protein gene (wsp) and Wolbachia hemo biosynthetic pathway gene (hemE) showed that these genes were strongly expressed in early embryos but not in later stage embryos (‘comma’, ‘pretzel’, or stretched intruterine microfilariae). A detailed study of the distribution of Wolbachia in B. malayi embryos using ISH with the 16S RNA probe documented the asymmetric segregation of Wolbachia in early embryos (morulae) and showed that Wolbachia were only present in hypodermal cord precursor cells in later stage embryos (‘comma’ stage or later). Progressively restricted localization of Wolbachia during morphogenesis may explain why Wolbachia are absent from the genital primordium in L3.

Wolbachia distribution and gene expression vary dramatically during filarial embryo development and across the worm’s life cycle. Additional research is needed to understand the tissue-specific population dynamics of Wolbachia and to explore how signals from the nematode host might influence the growth, distribution, and gene expression in these bacteria.

Keywords: Brugia malayi; Wolbachia; Embryogenesis; in situ hybridization; Gene expression

Introduction

Wolbachia are α-proteobacteria that are maternally transmitted and widely distributed (as an intracellular symbiont) in insects and in filarial nematodes. It is estimated that up to 65% of insect species harbor Wolbachia [1], and most filarial nematode species are infected with these bacteria [2]. Wolbachia infection is associated with several phenotypes in arthropod hosts such as cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing [3,4]. In contrast, many filarial nematodes require Wolbachia for development and reproduction [2].

These include Wuchereria bancrofti, Brugia malayi, and Onchocerca volvulus, which cause the disabling tropical diseases of lymphatic filariasis and onchocerciasis, respectively. These parasites depend on Wolbachia endosymbionts for their development, viability and fertility [2]. This dependence makes Wolbachia an interesting target for development of new drugs for lymphatic filariasis and onchocerciasis [5,6]. Animal studies and clinical trials have shown that doxycycline and rifampin therapies clear Wolbachia from filarial worms and inhibit fertility and larval development [7,8]. However, since doxycycline treatment is not practical for widespread use in tropical populations, researchers are searching for other drugs that can more efficiently kill Wolbachia in filarial worms [6]. A more comprehensive understanding of interactions between Wolbachia and their nematode hosts could lead to identification of new drug targets.

Wolbachia are present in all life stages in Wolbachia-dependent filarial species. However, the quantity and distribution of Wolbachia vary dramatically during the parasite life cycle [9,10]. The distribution of Wolbachia in adult filarial worms is well-documented, with the bacteria being concentrated in the lateral cords and the female reproductive system but not in the male reproductive system [11-13]. Wolbachia are asymmetrically separated into different cell lines during early embryogenesis, and this may explain the localization of the bacteria in adult worms [14].

This study employed in situ hybridization (ISH) to detect Wolbachia and to assess the expression of Wolbachia genes within the filarial host. The results include new information on the distribution of Wolbachia and changes in Wolbachia gene expression during embryo development.

Methods

Parasite material and in situ hybridization

B. malayi adult worms and L3 larvae were provided by the Filarialis Research Reagent Resource Center (FR3). Details regarding

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pathway gene hemE (Q5GTT) were amplified with these primers: 16S rRNA forward primer, 5-CAGCTCGTGTCGTGAGATGT-3, 16S rRNA reverse primer: 5-CCCAGTCACTGATCCCACTT-3, wsp forward primer: 5-TTTTTCAGCAACCGCCTTGTAG-3, wsp reverse primer: 5-ATTTACGCTATTCAGCTTCTG-3, hemE forward primer, 5-TGAAATTCTCGGTGCAAGT-3, hemE reverse primer: 5-CCTCCTGTGTCGCTTCA-3. The amplified fragments were subcloned into PCRII vector (Invitrogen, Carlsbad, CA), and purified plasmids were linearized for RNA probe synthesis. DIGoxigenin labeled RNA probes, sense (negative control for ISH) and anti-sense were transcribed in vitro with Sp6 and T7 RNA polymerases (New England Biolabs, Ipswich, MA). No signal was observed with control “sense” probes.

**DAPI staining**

After ISH, slides were washed in 1 x PBT (PBS plus 0.1% Triton) for 5 min and incubated with DAPI (8 µg/ml in PBT) for 5 min at room temperature (RT) followed by 2 x 5 min washes in PBT. Slides were sealed with ProLong gold anti-fade reagent (Invitrogen) and examined with a fluorescence microscope Olympus BX40 (Olympus, Center Valley, PA).

**Cell membrane staining**

Cell membranes were stained with wheat germ agglutinin (WGA) conjugated with Alexa fluor 633 (Invitrogen). After ISH, slides were washed in PBS for 5 min and incubated with WGA (1:200 in PBS from 1 mg/ml stock solution) for 10 min at RT, followed by 3 x 5 min washes at RT. Slides were sealed with ProLong gold anti-fade reagent (Invitrogen) and reviewed by fluorescence microscopy.

**Photo modification**

Acquired photos were merged as layers with Adobe Photoshop CS2 (Adobe, San Jose, CA).

**Results**

**Detection of Wolbachia and Wolbachia gene expression by in situ hybridization**

Large amounts of *Wolbachia* were detected in adult *B. malayi* worms by ISH with the *Wolbachia* 16S rRNA probe. *Wolbachia* were detected in the lateral cords in adult male and female worms (Figure 1, Figure 2). However, the bacteria were unevenly distributed along the cords. In some sections, *Wolbachia* were detected throughout both lateral cords (Figure 1A, Figure 2A). In others, *Wolbachia* were only observed in one cord (Figure 1C, Figure 2C) or only in some segments of one cord (Figure 1B, Figure 2B). *Wolbachia* were only observed in lateral cords in males (Figure 1), but the bacteria were also seen in oocytes and all stages of developing embryos in female worms (Figure 2).

As in adult males, *Wolbachia* were only observed in hypodermal cords in third stage infective larvae (L3) which include both sexes (Figure 3).

*Wolbachia* surface protein is a widely used target for immunohistological detection of *Wolbachia*. The expression pattern of wsp was somewhat different from that of 16S rRNA gene. ISH signals for wsp were detected in lateral cords in adult male and female worms, and wsp expression was also detected in oocytes and in early embryos (morulae) in female worms. However, in contrast to 16S, wsp was not expressed in late embryos (pretzel and stretched microfilariae stages) (Figure 4). The heme biosynthetic genes of *Wolbachia* are considered
Distribution of Wolbachia during embryogenesis

We examined the distribution of Wolbachia in adult female reproductive organs by ISH with 16S rRNA probe. Wolbachia were diffusely present in unfertilized oocytes in ovaries (Figure 6A). In fertilized oocytes, Wolbachia segregate to the poles prior to the first cell division (Figure 6B). As early as the first cell division, Wolbachia were asymptomatically separated into precursors of the soma and germ line [14]. Early divisions after the first cell division produce early morula stage embryos as the somatic and the germ line precursors further differentiate into various cell lines [14]. In early morulae, the Wolbachia were asymmetrically segregated during cell division, and variable numbers of Wolbachia were distributed into different daughter cells (Figure 6C). However, some Wolbachia were still present in most cells at this time. In late morula embryos more cells lack Wolbachia, and the quantity of Wolbachia in different embryos appeared to vary (Figure 6D), although this could be an artifact related to sectioning. Wolbachia were absent from most cells in comma stage embryos, and the bacteria were mostly confined to cells on the edge of the embryos that probably are precursors of hypodermal cords (Figure 6E). In later pretzel stage embryos, Wolbachia were clearly localized in one or two longitudinal dotted lines along the edges of the embryos (Figure 6F). This pattern was also observed in mature embryos (stretched microfiliariae inside adult females) (Figure 6G) and in L3 (Figure 3).

Discussion

Prior studies have used methods such as PCR, immunohistology (IH), and electron microscopy (EM) to study Wolbachia in filarial worms. Each method has advantages and disadvantages. PCR is simple and very sensitive, but it does not provide information on localization of Wolbachia in the worms. IH provides localization information, but it does not provide direct information on gene expression or viability of the bacteria. Also, some of the antibodies used for IH produce significant background staining, because they also label mitochondria (e.g. anti-HSP60, anti-GroEL) [17,18]. EM is useful for visualizing
**Figure 6:** Wolbachia are asymmetrically segregated into different cells during early embryogenesis and are eliminated from all cells except hypodermal cord cells during morphogenesis. Wolbachia were labeled by ISH using a 16S rRNA probe (left panel), and DNA was stained with DAPI (blue, right panel). Developing oocytes in ov1 are more advanced than in ov2 and embryos in ut1 are more advanced than in ut2. (A) Wolbachia are present in most of the developing oocytes in ovaries; (B) Wolbachia moved predominantly to the poles after fertilization; (C) in early morula stage, Wolbachia are asymmetrically segregated in many blastomeres; (D) in the late morula stage, Wolbachia are present in a smaller number of cells; (E) in comma stage embryos, Wolbachia are absent in most cells and concentrated in hypodermal cord cells; (F) in pretzel stage embryos, Wolbachia were only detected in hypodermal cord cells; (G) in stretched microfilariae, Wolbachia were only visible in hypodermal cord cells. Abbreviations: i: intestine; lc: lateral cord; ov: ovary; ut: uterus; scale bar: 20 µm.

Wolbachia, but it is difficult to get an overview of their distribution in different parts of the worms with this technique. The present study used ISH to detect Wolbachia in filarial worms. ISH localized the Wolbachia in tissues and organs of the worms as well as IH. ISH results with a 16S rRNA probe showed that Wolbachia were present in the lateral cords in adult male and female *B. malayi* worms, and in oocytes and embryos in females. These results showed that in situ can be used as an alternative to other methods for localizing Wolbachia. Our results confirm prior results obtained by IH and EM [11,13,17,19], and this consistency helped to validate the ISH method. Advantages of in situ are that it provides information on bacterial viability and gene expression [20]. Wolbachia of filarial parasites cannot be cultured or easily isolated from host tissue. Therefore, expression analysis of Wolbachia genes is sometimes difficult because the amount of Wolbachia mRNA is often very low relative to mRNA transcribed from the nuclear genes. ISH hybridization offers the opportunity to study Wolbachia gene expression within the bacterial cells and in relation to the infected host tissue. The ISH probes used in this study were specific for Wolbachia; ISH signals were not observed in *B. malayi* tissues that do not contain Wolbachia.

The distribution of Wolbachia in adult male somatic tissues (lateral cords) in *B. malayi* worms is different from their distribution in *Drosophila* and other insects. For example, Wolbachia are sometimes present in germline stem cells in male *Drosophila*; they are also present in cells involved in spermatogenesis, and only discarded in elongated sperms [4]. In the wasp *Nasonia vitripennis*, Wolbachia are observed throughout the whole testis and present in 28% of developing spermatocytes [21].

Although, we are not able to distinguish future males from females in sections of filarial L3, we did not observe Wolbachia outside of the hypodermal cords in sections that sampled at least 1000 L3 larvae. This suggests that Wolbachia are not present in germline precursors in L3, and this would be consistent with their later absence in the reproductive system of male worms.

ISH showed that Wolbachia were not evenly distributed in lateral cords of adult worms. This uneven distribution of Wolbachia in lateral cords has also been observed by IH staining [14]. It is unknown whether Wolbachia can spread between syncytia of the lateral cord, although it has been suggested that Wolbachia may use cell fusion to populate the hypodermal cords [14].

In addition to studies with the 16S rRNA probe, we also performed ISH with a probe for the gene wsp. ISH results with the wsp probe were mostly similar to results obtained by ISH with a 16S rRNA probe and by WSP IH staining [10,17]. All of these methods label Wolbachia in lateral cords, oocytes and early embryos. However, unlike the 16S rRNA probe and WSP IH results, the wsp rRNA probe did not label late stage embryos (pretzel stage larvae and stretched microfilariae). In late stage embryos, wsp may not be actively transcribed (therefore, not detected by wsp ISH), but WSP proteins are still present in the cells (detectable by WSP IH). This suggests that wsp gene expression is down-regulated in stretched mf and that Wolbachia gene expression varies during embryo development. Thus, besides providing information on localization and the concentration of Wolbachia in parasite tissues, ISH can be used to study the expression of individual Wolbachia genes during embryo development and in different stages in the life cycle of the parasite.

The population dynamics of Wolbachia in *B. malayi* has been investigated by quantitative PCR which demonstrated changes in Wolbachia DNA content in different life stages of the parasite [9,10]. A recent study used IH staining to study the distribution of Wolbachia in early embryogenesis [14]. The present study used ISH to assess Wolbachia during embryogenesis in *B. malayi* worms. We found that Wolbachia were present in almost all developing oocytes; this result was similar to that reported for *Drosophila* (transmission rate 97%) [22-24]. ISH showed that Wolbachia were concentrated at the poles of fertilized oocytes. Landman et al. [14] reported that subsequent cell divisions establish the somatic blastomere AB and the germ line blastomere P1 which further divided into ABa, Abp and MS, E, C, D, P4.
blastomeress, respectively [14]. Our results confirm that Wolbachia are asymmetrically distributed into the AB and P1 blastomeres and their descendent blastomeres.

As embryos developed to the comma stage, Wolbachia became concentrated on the edge of the embryos and lined up in a curved structure. In later embryos (pretzel and stretched microfilariae), Wolbachia were only observed along two longitudinal dotted lines that probably correspond to developing hypodermal lateral cords. Wolbachia were not seen in other cells in these stages. Wolbachia distribution patterns in pretzel stage embryos and in stretched intrauterine microfilariae were similar to those seen in L3 larvae. Therefore, Wolbachia seem to be eliminated from all cell types other than hypodermal cord cells as embryos develop from the comma to the pretzel stage.

We do not know how Wolbachia are eliminated from some cell lines. One possible mechanism is dilution during cell proliferation. During early embryogenesis, Wolbachia numbers are fairly constant (approximately 70 per embryo) as the number of nematode cells increases from one to more than 100 per embryo [14]. Due to the asymmetric segregation of Wolbachia, the bacteria become concentrated in cells of blastomere P1 origin. The mechanism of this segregation is not understood. However, dilution does not explain the absence of Wolbachia in germline precursor cells in late stage embryos, L3, and adult males, since germline precursor cells contain many Wolbachia during early embryogenesis. More research is need on this topic.

In conclusion, filarial worms require Wolbachia for reproduction and development, and clinical trials have shown that Wolbachia represent an ‘Achilles heel’ for these worms. Further understanding of the Wolbachia-worm relationship may help us to better exploit this vulnerability. This study has contributed to this effort by demonstrating that ISH can provide interesting new information on the modulation of Wolbachia numbers, distribution, and gene expression in filarial worms during embryo development and across the life cycle.

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