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OPTIMIZATION OF METHODS FOR CROSS-SPECIES INFECTION OF CELL CULTURES WITH *WOLBACHIA*

By

Sarah Lane

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ABSTRACT

Optimization of methods for cross-species infection of cell cultures with *Wolbachia*

Sarah Lane

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The common intracellular endosymbiont genus of bacteria called *Wolbachia* is of interest due to the promise it shows for playing a role in disease control. Wolbachia have a number of widely variable effects on its many species of host insects. In combination with these roles, Wolbachia largely affects reproduction and development of its host species. It can lead to feminization and cytoplasmic incompatibility (Werren et al. 2008). Furthermore, this species is vertically transmitted which means that it passes from mother to offspring (Caragata et al., 2016). In combination with its effects on reproduction, which can allow *Wolbachia* to propagate through a species following its introduction, the role that *Wolbachia* play on immunity to pathogens are what has caused it to be considered as a possible biological tool to minimize disease transmission to human populations. The current study sought to improve lab techniques as they can be used to study *Wolbachia* through infection of cell cultures. In this study, cell lines of fruit flies, or *Drosophila melanogaster*, were cultivated and infected with Wolbachia isolated from bed bugs (*Cimex lectularius*). Microscopy was used to look for indications of desired Wolbachia infection. Results of this study include determination of optimal centrifuge settings and dye technique for this process as well as the necessity of filter use. Finally, the study concluded that juvenile/nymph bed bugs were most useful for *Wolbachia* extraction.

KEYWORDS: *Wolbachia*, Infection, Disease control, Fruit flies, *Drosophila melanogaster*, bed bugs, *Cimex lectularius,* Cell cultures, Methods, Microscopy, Punctae

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CHAPTER 1

Introduction

Bacteria in the genus *Wolbachia* are common intracellular symbionts among insect species (Werren et al. 2008). Approximately 52% of insect species are infected with *Wolbachia* to some extent (Sazama et al., 2017). However, despite this large rate of infection across species, generally, infection prevalence varies between species (Sazama et al., 2019). Previous research suggests that this symbiotic relationship has many different effects on individuals within insect populations, including positively influencing survival of the host insect. This can result from the roles *Wolbachia* plays in regulating immunity to pathogens, metabolism, development, and other physiological processes. For example, *Wolbachia* is known to affect iron metabolism of insects (Kremer et al. 2009) as well as lipid metabolism in mosquitos (*Aedes albopictus*) (Malloy et al. 2016). *Wolbachia's* major effects on reproduction include feminization of individuals and cytoplasmic incompatibility (Werren et al. 2008). Due to *Wolbachia's* ability to affect the physiology of insects that vector disease-causing microorganisms, researchers are currently studying and considering its use for the purpose of disease control, especially in common carriers of deadly diseases like mosquitoes that transmit diseases such as dengue and zika virus. This solution could be very effective in minimizing and even eradicating disease transmission by insects. Additionally, because *Wolbachia* is vertically transmitted during reproduction, this possible disease control measure has the potential to naturally spread across insect populations, boosting its promise (Caragata et al., 2016).

However, before this ultimate goal of utilizing *Wolbachia* infection for the purpose of disease control can be achieved and efficiently utilized, methods to artificially (non-naturally) infect insects with *Wolbachia* must be further refined. These methods have the potential to facilitate the practical use of *Wolbachia* infection for the purpose of preventing disease transmission from various insects to humans. Further, additional information on the cellular effects and transmission mechanisms of *Wolbachia* will also be useful in refining this approach.

The current study aimed to analyze various methods for achieving cross-species infection of insect cell cultures with *Wolbachia.* As a proof-of-concept, *Wolbachia* was isolated from bed bugs (*Cimex lectularius*) and transferred to cell cultures derived from the fruit fly (*Drosophila melanogaster*). Bed bugs were chosen for this purpose due to their "bacteriocyte-associated obligate nutritional mutualist" *Wolachia* (Slatko et al., 2010). In other words, since bed bugs cannot live without *Wolbachia*, all bed bugs have intracellular *Wolbachia* which is convenient for this study. The recipient of the isolated *Wolbachia* in this study was a cell line generated from *Drosophila* which is both a common host for intracellular bacteria and manageable cell in the laboratory as noted by Luce-Fedrow et al. 2014. Isolation methods performed and analyzed in this study included different procedures to extract *Wolbachia* from either adult or nymph (immature) bed bugs. Additionally, the use of centrifugal filter columns for *Wolbachia* extraction was also examined. It is commonly known that *Drosophila* are tremendously useful and convenient in the lab, especially for genetic studies. In the case of this study, characteristics of *Drosophila* such as easy attainability, cost-effectiveness, and high susceptibility to bacterial infection were major factors in the choice to use *Drosophila*

cell lines. Analysis of success for the various infection methods was done via microscopy of stained recipient cells that allowed visualization of both the *Drosophila* cells as well as the desired intracellular *Wolbachia* bacteria when applicable.

CHAPTER 2

Materials & Methods

Generating and cultivating S2 cells lines

In order to nurture a cell line for the purpose of *Wolbachia* infection, we first started with frozen S2 cells, letting them defrost. After transferring S2 cells to a 1.5 mL tube, 1mL of cell culture medium (Expression systems ESF 921) was added. The tube was then centrifuged at 1000 relative centrifugal force (rcf) for 5 minutes. Following this, the supernatant on top of the tube was decanted, leaving only the pellet of S2 cells behind. Next, 1 mL of cell culture medium was added to the tube which was then mixed using a Fischer Scientific vortex mixer in order to disturb the cell pellet, causing it to go into solution. Following this, a T25 tissue culture plate was prepared by adding 4 mL of cell culture medium followed by the contents of the tube. This tissue culture plate was then incubated at 28°C and monitored for desired growth. In the case of this study, about two to four days of growth was sufficient. Any more than that typically required either the cell medium to be changed in order to replenish the S2 cells or for the S2 cells to be split into another flask, also providing replenished cell culture medium.

In order to prepare S2 cells for *Wolbachia* infection, cells were transferred to a 12-well cell culture plate. Once sufficient growth of cells incubated on the T25 tissue culture plate was observed, this transfer was then conducted. First, a glass scraper was used to physically disrupt cells adhered to the T25 tissue culture plate. Next, the contents of this tissue culture plate were transferred to multiple tubes which were then centrifuged at 1000 rcf for five minutes. Again, supernatant from the tops of these tubes was decanted followed by replenishment of cell culture medium, and mixing on the vortex mixer. After putting a single microscope coverslip at the bottom of each well to be used, solutions in the tubes were then distributed evenly among the wells. The 12-well cell culture plate was then incubated at 28 degrees Celsius to allow both growth and adhesion of cells to available surfaces.

Isolation of Wolbachia from bed bugs & infection of S2 cells

Method #1: from adult bed bugs

The methods used by Frydman 2006 for isolation of *Wolbachia* from adult fruit flies were adapted slightly for *Wolbachia* isolation from adult bed bugs. First, adult bed bugs were collected and washed in 70% alcohol. After the alcohol was dried, a single leg was removed from each insect, and the bed bugs were then put in a centrifugal filter column with pores of 65 µm and centrifuged at 5000 relative centrifugal force (rcf) for five minutes. This process allows *Wolbachia* infected cells to be extruded from the body while also rupturing the cells and releasing free *Wolbachia*.

Method #2: from nymph bed bugs

This procedure began with collection of juvenile (nymph) bed bugs which were washed in a 1.5 mL tube of 70% alcohol. This tube was then centrifuged at 500 rcf for three minutes, but this speed proved unsuccessful to pellet the insects so a revised centrifuge speed and time of 1000 rcf for five minutes was used from then on out. Following centrifugation, alcohol was removed from the tube using a pipette and remaining alcohol was given time to evaporate before proceeding. Following this, a small amount of cell culture medium, between 100 to 300 μL, was added before using a pestle to crush bed bugs and release *Wolbachia* present in tissues and hemolymph. After crushing bed bugs, the solution appeared murky due to the release of cells. After letting debris such as fragments of insect cuticle settle to the bottom of the tube, S2 cells cultured in a 12-well plate as described above were then infected by adding about 100 μL of tube contents (i.e. solution with *Wolbachia* from bed bugs) to each cell well in use, leaving one well as an uninfected control. 1 mL of cell culture medium containing 2% penicillin-streptomycin antibiotic was then added to each well. This solution served to nurture cells while also preventing the growth of contaminating bacteria but allowing *Wolbachia* to grow, as it is not susceptible to these antibiotics (Hermans et al. 2001). Finally, the 12-well cell culture plate was again incubated at 28 degrees Celsius for both cell growth and to allow intracellular infection to occur. Because *Wolbachia* are intracellular symbionts, it is expected that infection takes place without an environmental or chemical catalyst (White et al., 2017).

Method #3: from nymph bed bugs using filter

Ultimately, this procedure was similar to Method #2, but included an additional centrifuge step after crushing of the bed bugs with a pestle. In this step, centrifugal filter columns were used as in Method #1. This was to enhance the *Wolbachia* isolation and reduce toxic debris.

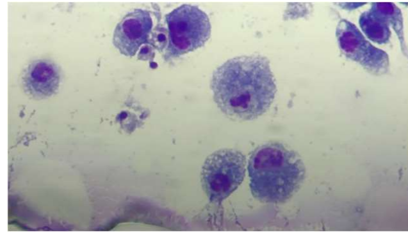
Staining cells for visualization of Wolbachia

This procedure can be used for any cells adhered to a microscope coverslip that investigators may wish to view or analyze. While cell growth may be examined in either the T25 cell tissue flask or the 12-well cell culture plate without any staining techniques, this method differentially stains cellular components and bacteria and is necessary to determine if desired *Wolbachia* infection is present. After ensuring sufficient cell growth, a microscope cover slip containing cells was removed from the 12-well plate, being careful to maintain sterility of the remaining wells through use of the sterile hood. The microscope coverslip to be stained does not require a sterile environment directly before staining so its removal from the hood is acceptable. In this process, the microscope coverslip was first dipped briefly 5 to six times into solution number one (*Methanol fixative*) of a KWIK-DIF stain set followed by dabbing excess solution onto a paper towel. This procedure proceeded similarly, dipping the microscope coverslip into solution number two (*Eosin*) ten times, followed by solution number three (*Methylene Blue*) only four or five times, being sure to remove lingering solution onto a paper towel. Next, the

slide was rinsed by sipping it in water. Finally, the slide was allowed time to air dry before analysis under the microscope.

CHAPTER 3

Results



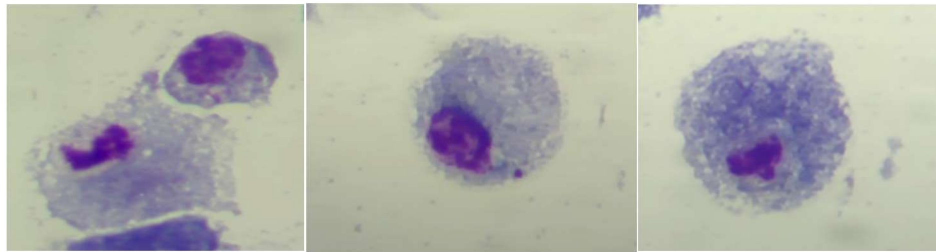


Fig. 1. Representative images of uninfected S2 cells control, 1000X magnification

Examination of uninfected S2 cells and their growth can be seen via microscopy in **Figure 1**. In this study, we were successful in cultivating a usable cell line of S2 of which was then used for *Wolbachia* infection and analysis of that infection. In **Figure 1**, cell nuclei were differentially stained darker due to methods outlined above while the cytoplasm appeared lighter and smooth without significant punctae. These cell cultures serve the purpose of a control for comparison for the rest of the experiment and *Wolbachia*-infections that were performed.

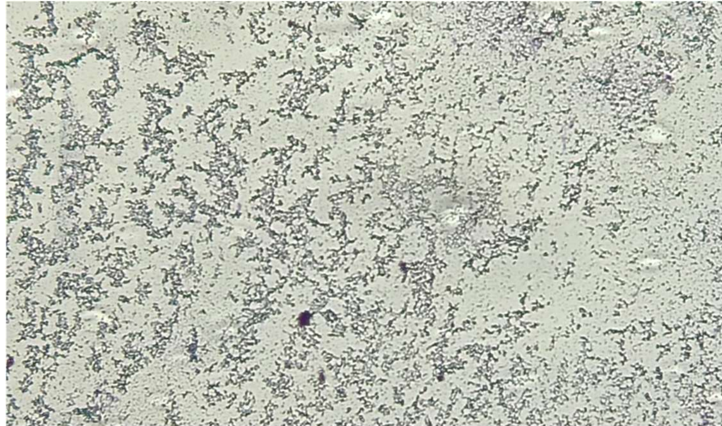


Fig. 2. Representative image of *Wolbachia* preparation extracted from crushed and filtered bed bugs, 1000X magnification

Much like the nuclei of cells, bacteria also take a darker color than the cell cytoplasm during the staining procedure we used. In **Figure 2**, extracted bacteria (*Wolbachia*) from crushed and filtered bed bugs (method #3), along with cellular remnants of bed bugs can be seen. Importantly, however, large intact cells cannot be seen, indicating the success of the extraction procedure. Like **Figure 1**, this image also serves as a control for procedures in which S2 and *Wolbachia* were viewed in the same microscope slide.

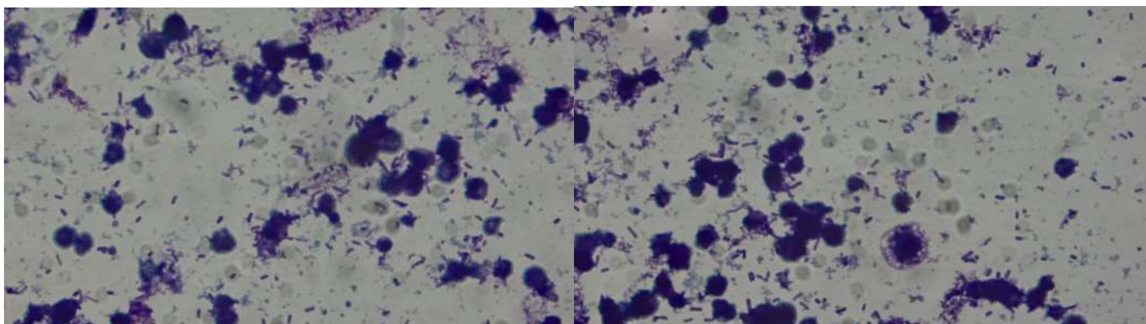


Fig. 3. Representative images of S2 cells after addition of crushed bed bug nymphs with no filtering (method #2), 1000X magnification

The images in **Figure 3** depict bacterial infection of S2 cells in which a filter was not used during the *Wolbachia* isolation from juvenile bed bugs. These are of concern, however, because along with visible mortality in the S2 cells indicated by shrinking and rupturing, a large number of extracellular bacteria are also visible. In the case of *Wolbachia* infection, the *Wolbachia* bacteria, which are naturally intracellular will inhabit cells rather than remain in the extracellular environment. Therefore, these findings are indicative of a possible unintended/uncontrolled bacterial contamination. These results are likely a result of lack of filter use during *Wolbachia* isolation. However, the intracellular bacteria visible may be *Wolbachia*.

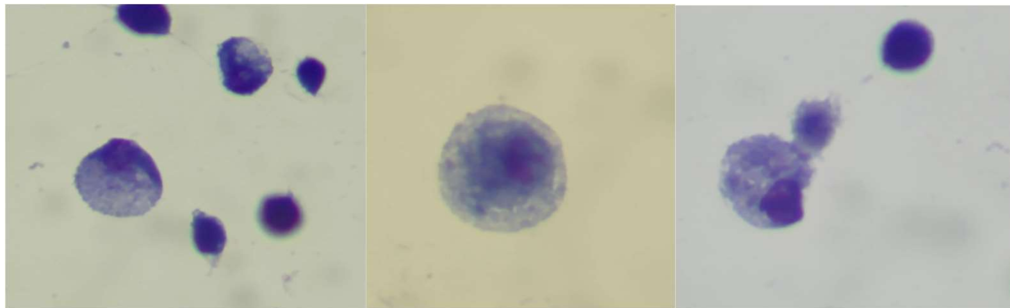


Fig. 4. Representative images of S2 cells after addition of crushed bed bugs with filtering (method #3), 2 days post-infection, 1000X magnification

Figure 4 shows results of infection attempts of S2 cells using method #3. Comparison of **Figure 4** to the control, **Figure 1**, a difference could be seen by 2 days post-treatment with *Wolbachia* extract. Unlike cells of **Figure 1**, which depict a distinct, identifiable nucleus and a smooth lightly colored cytoplasm, some cells depicted in **Figure 4** had numerous dark cytoplasmic punctae. This is indicative of desired *Wolbachia* infection, but only a small proportion of cells examined at this time showed this phenotype.

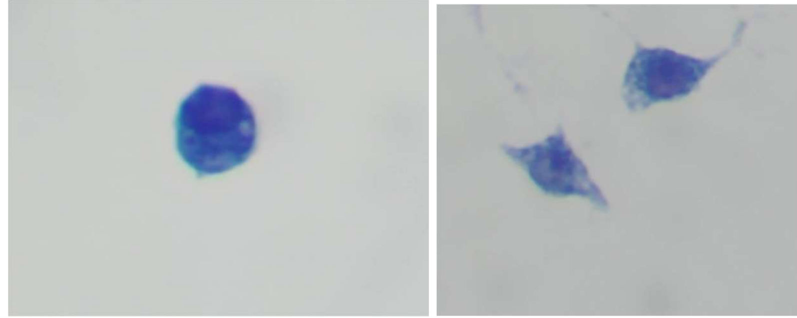


Fig. 5. Representative images of S2 cells after addition of crushed bed bugs with filtering (method #3), 7 days post-infection, 1000X magnification

On examination of S2 cells 7 days post-treatment with Wolbachia extract (method#3) (**Figure 5**), the prevalence of cells with punctae appeared to increase. This was consistent with examination at 2 days and suggested that intracellular *Wolbachia* infection had in fact taken place.

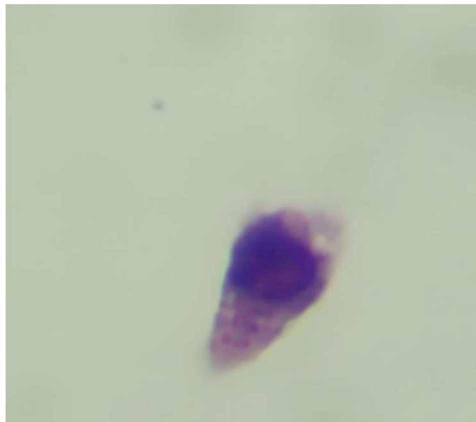


Fig. 6. Representative image of S2 cells after addition of crushed bed bugs with filtering (method #3), 9 days post-infection, 1000X magnification

Microscopy of S2 cells 9 days post-treatment with *Wolbachia* extract (method #3) (**Figure 6**) yielded similar results to those on day 7. Images of cells with similar punctae

can be seen, but the prevalence of cells with these punctae was perhaps less than in the previous analysis done on 7 days post-treatment (**Figure 5**).

CHAPTER 4

Discussion

The current study aimed to examine various *Wolbachia* isolation techniques as well as their applicability and viability in regards to infection of S2 cells. Key findings are regarding optimal life-stage of bed bugs to be used, filter use, centrifuge speed, and dying technique. First, isolation of *Wolbachia* from adult bed bugs was unsuccessful to the extent that no *Wolbachia* was seen on various microscope coverslips using microscopy and this method also appeared to have some toxicity against the S2 cells. Therefore, we suggest use of juvenile bed bugs for *Wolbachia* isolation. Next, although *Wolbachia* infection from juvenile bed bugs appeared successful, when a filter was not used, bacteria-crowded S2 cells were seen. It is likely that lack of filter use allowed bacteria other than *Wolbachia* bacteria to stay with the solution of the centrifuged test tube. Therefore, we determined that filter use of the appropriate size is essential. This study also served to indicate appropriate centrifuge speed and time. Finally, after trying various combinations of dying techniques with a KWIK-DIFF dye set, the optimal combination was determined as outlined in *Staining cells for visualization of Wolbachia.*

Further findings of this study suggest interesting future directions. In this study, change of infection over time was assessed via microscopy on 2 days, 7 days, and 9 days post-treatment. Since the prevalence of punctae on day 7 post-infection appeared significantly greater on 2 days post-treatment, it appears that optimal *Wolbachia* infection may take a number of days. However, infection rates appeared similar if not waning on 9 days post-infection so it is still uncertain how long infection can last or if infection is stable and propagating as it would in *vivo*. Future studies should quantitatively monitor for signs of infection over a longer period of time. Performing more quantitative studies of infection changes over time would serve to confirm our suspicions regarding *Wolbachia*'s stability and propagation over time.

Finally, the methods of this study, though conventional, were crude and preliminary. As a result, we suggest more intensive methods to confirm *Wolbachia* infection as the methods used in this study did not explicitly prove that *Wolbachia* was the bacteria infecting S2 cells. This was only inferred due to the expected visual phenotype of the cells and lack of other intracellular bacteria besides *Wolbachia* in bed bugs. To confirm *Wolbachia* infection, molecular methods such as PCR would work well as discussed in Simões et al. (2011).

Overall, this study provides a good foundation for future laboratory studies to isolate and compare different strains of *Wolbachia* in cell culture. The fundamental information obtained in this study could eventually be useful to researchers in establishing cross-species infection for disease vector control.

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