Wolbachia isolation by fractioned centrifugation from *Dirofilaria immitis* for protein analysis

Vivornpun Sanprasert*
Sivapong Sungpradit*; **Surang Nuchprayoon*


**Objective**

The biological roles of Wolbachia, a filarial intracellular bacteria, have promoted applied researches in lymphatic filariasis. Analysis of Wolbachia proteins will provide complementary information on the biology of Wolbachia, as well as the immune mechanisms against filarial nematodes. We therefore developed a Wolbachia isolation method from *Dirofilaria immitis* (dog heartworm) for protein analysis.

**Methods**

Wolbachia bacteria were homogenized and isolated by fractioned centrifugation by using 0.85% NaCl supplemented with varying concentration of Nonidet P-40 (NP-40) as the homogenization buffer. The purity of Wolbachia extracts were analyzed by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), and by quantitative polymerase chain reaction (qPCR) targeting the single copy of the Wolbachia surface protein (wsp) gene. The contaminations from filarial extracts were analyzed by qPCR targeting the filarial glutathione-S-transferase (gst) gene.

**Results**

We were able to isolate Wolbachia bacteria from *D. immitis* with high purity. The higher concentration of NP-40 could increase the yield of bacterial isolation and decrease the host parasite contaminations.

* Lymphatic Filariasis Research Unit, Department of Parasitology, and Chulalongkorn Medical Research Center, Faculty of Medicine, Chulalongkorn University
**Department of Pre-clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University
Conclusion : The homogenization buffer which provided the highest Wolbachia protein concentration, but the lowest filarial contamination, was 0.85% NaCl + 0.08% NP-40. This technique is likely to provide the most suitable methods for Wolbachia isolation to facilitate the proteomic studies. However, our purification procedures do need validation in other applications, which require intact Wolbachia DNA materials, and/or viable cells.

Keywords : Wolbachia isolation, Dirofilaria immitis, Protein analysis.
วิวรพรรณ สรรประเสริฐ ศิริพงษ์ สิทธิประยูร สุรางค์ นุชประยูร การแยกแบคทีเรียโวลบาเชียจากหนอนพยาธิหัวใจสุนัขโดยวิธีปั่นแยกลำดับขั้นสำหรับการศึกษาโปรตีน
จุฬาลงกรณ์เวชสาร 2553 พ.ย. - ธ.ค.; 54(6): 537 – 47

วัตถุประสงค์ : บทความทางวิชาวิทยาของแบคทีเรียโวลบาเชียสนับสนุนให้มีการศึกษาวิจัย ปรากฏในโรคเท้าช้าง การวิเคราะห์โปรตีนของแบคทีเรียโวลบาเชียจะให้ ข้อมูลที่ดีในหลายชีววิทยาของแบคทีเรียโวลบาเชียรวมถึงการตอบสนอง ทางภูมิคุ้มกันหยุดพยาธิพิษต่อการ คณะผู้วิจัยจึงพัฒนาวิธีการแยกแบคทีเรียโวลบาเชียจากหนอนพยาธิหัวใจสุนัขสำหรับการศึกษาไวยาบันทึก

วิธีการ : แบคทีเรียโวลบาเชียถูกแยกโดยวิธีปั่นลำดับขั้น โดยใช้ 0.85% NaCl ผสมด้วย Nonidet P-40 (NP-40) ที่ความเข้มข้นต่าง ๆ เป็นสารสกัดพื้น ความบริสุทธิ์ของ แบคทีเรียโวลบาเชียที่สกัดแยกได้ถูกวิเคราะห์โดยวิธี sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) และวิธี quantitative polymerase chain reaction (qPCR) โดยใช้ยีนเป้าหมายเป็นยีนที่มีเพียงยีนเดียวในเซลล์ของแบคทีเรียโวลบาเชียคือยีน glutathione-S-transferase (gst)

ผลการทดลอง : คณะผู้วิจัยสามารถแยกแบคทีเรียโวลบาเชียจากหนอนพยาธิหัวใจสุนัขได้ด้วยความบริสุทธิ์สูง โดยความเข้มข้นของ NP-40 ที่เพิ่มขึ้นสามารถเพิ่มปริมาณแบคทีเรียโวลบาเชียที่แยกได้เพิ่มขึ้น และสามารถลดการปนเปื้อนจากหนอนพยาธิ ซึ่งเป็นโฮสต์ได้

สรุปผล : สารสกัดพื้นที่ให้ความเข้มข้นของโปรตีนของแบคทีเรียโวลบาเชียมากที่สุด แม้ในการปนเปื้อนจากโปรตีนของหนอนพยาธิหัวใจสุนัขเพิ่มขึ้น 0.85% NaCl + 0.08% NP-40 วิธีการแยกที่ได้ผลลัพธ์ที่เหมาะสมสำหรับการแยกแบคทีเรียโวลบาเชีย เพื่อการศึกษาไวยาบันทึกแบคทีเรียโวลบาเชีย บางมีเชื้อในวิธีการแยก ปนเปื้อนที่น่าจะเป็นต้องพิสูจน์ความเหมาะสมสำหรับการใช้ในงานวิจัย ซึ่งต้องใช้สารพันธุกรรมที่สมบูรณ์ของแบคทีเรียโวลบาเชีย และ/หรือเซลล์ที่มี ชีวิตต่อไป

คำสำคัญ : การแยกแบคทีเรียโวลบาเชีย, หนอนพยาธิหัวใจสุนัข, การวิเคราะห์โปรตีน
The obligate intracellular bacteria of the genus *Wolbachia* has been discovered in arthropods and filarial parasites. *Wolbachia* is alphaproteobacteria belonging to the order Rickettsiales, and family Anaplasmataceae.\(^1\) It is described by the general characteristics of rickettsiae; small (0.3-0.5 x 0.8-2.0 μm) gram-negative coccobacilli that is obligate intracellular bacteria of eukaryotic cells. Under electron microscopy studies, the bacteria typically reside within a vacuole covering with three layers of membranes in the cytoplasm of the cell. The outer layer is host-derived, followed by the outer cell wall of the bacteria, and the innermost layer consists of the plasma membrane of the bacteria.\(^2,3\)

*Wolbachia* appears to play an important role in the development, viability and fertility of filarial nematodes. The significant biology functions of *Wolbachia* have promoted applied researches to use them as targets for a novel chemotherapy in filarial disease control and eradication.\(^4-6\) *Wolbachia* also plays an important role in the pathogenesis of filarial diseases.\(^7,8\) A relevant study has suggested that *Wolbachia*, rather than the lymphatic filarial parasite *Brugia malayi*, plays a major role in inducing inflammatory responses by mouse macrophages.\(^9\) Host immune responses against *Wolbachia* are thought to be a major factor contributing to the progression of lymphatic filariasis, and drug-induced adverse reactions.\(^10-14\)

Analysis of *Wolbachia* proteins will provide complementary information on the biology of this endosymbiont, and a basis molecular mechanism of the inflammatory response in lymphatic filariasis. Because *Wolbachia* is an intracellular bacterium, the *Wolbachia* isolation from filarial parasites has been impeded by the difficulty to obtain the highly purified and sufficient amount of *Wolbachia*. To harvest *Wolbachia* protein materials for protein analysis, we therefore developed a method to isolate *Wolbachia* from filarial nematodes for protein analysis.

All extraction methods for isolation of rickettsia bacteria from host cells are not effective to isolate *Wolbachia* from *Drosophila* eggs.\(^15\) The major contaminations are mitochondria.\(^15\) Sun et al modified methods to purify mitochondria from *Drosophila* and isolate *Wolbachia* bacteria from adult worms of *B. malayi* by using 0.85% NaCl supplemented with 0.001% Nonidet P-40 (NP-40). This was a non-ionic detergent which was used as the homogenization buffer for determination of *Wolbachia* genome size as well.\(^16\) However, this isolation method was not proved to be suitable for proteomic studies.

Detergents are known as surfactants which decrease the surface water tension, and solubilize membrane proteins by mimicking the lipid-bilayer environment.\(^17\) Therefore, the higher percentage of non-ionic detergent should increase yield of the bacterial isolation as well as decrease host parasite contamination. In this study, we investigated the most appropriate concentration of NP-40 as homogenization buffer to isolate *Wolbachia* from *D. immitis*.

Therefore, we adjusted a proper condition for the *Wolbachia* isolation from *Dirofilaria immitis* by various concentrations of NP-40 ranging from 0.04% to 0.10% in the homogenization buffer.
Materials and Methods

Preparation of adult worm extracts

This study has been approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Adult worms of *D. immitis* were collected from hearts of naturally infected dogs at necropsy. Worms were washed with normal saline, and chopped up on ice with a sterile scalpel blade. The chopped-up worm tissue fragment of about 200 mg were homogenized in pestle homogenizer in ice-chilled with 1.2 ml of 0.85% NaCl supplemented with 0.04%, 0.06%, 0.08%, or 0.10% NP-40 (Sigma, St. Louis, MO) as the homogenization buffer.

Wolbachia isolation

The purification procedures for filarial *Wolbachia* were modified from the protocol previously described.\(^{(16)}\) After incubation on ice for 15 min *Wolbachia* cells were isolated by differential centrifugation (Figure 1). To pellet the worm debris and worm nuclei, the homogenates were centrifuged at 350 xg at 4°C for 40 minutes. To pellet *Wolbachia* bacteria, the supernatant was further centrifuged at 16,100 xg at 4°C for 20 minutes. The *Wolbachia* pellet was resuspended with the homogenization buffer. After three washes with the homogenized buffer, enriched *Wolbachia* pellet was collected, and examined under a light microscope.

Figure 1. The schematic diagram of the process used for *Wolbachia* isolation from *Dirofilaria immitis* adult worms.
**Wolbachia** protein analysis

Wolbachia proteins were characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the density values of the bands were analyzed by Quantity One Software version 4.6 (Bio-Rad Laboratories, Inc., Hercules, CA).

**Wolbachia** purification

The purity of the Wolbachia was determined by the ration of wsp/gst which characterized by quantitative real-time PCR. The method for Wolbachia detection using qPCR was modified from the protocol previously described. The Wolbachia pellets were suspended in 5 μl lysis buffer (0.05% SDS and 0.025N NaOH). The samples were incubated at 95°C for 15 min, spun down, and resuspended in 195 μl sterile deionized water. All DNA extractions were stored at -20°C until use. The quantitative real time PCR analysis was performed using specific *D. immitis* Wolbachia surface protein (wsp) primers, wspDiro-F (5’ CTT TAG TGG CGT TGC TAA GT 3’) and wspDiro-R (5’ ACC TTC AAT ATC AAC CCT AAT ATC G 3’) (Invitrogen, Carlsbad, CA). The amplification program included an initial denaturation step for 1 cycle at 95°C for 10 min and 45 cycles of denaturation at 95°C for 10s, annealing at 60°C for 10s, and extension at 72°C for 5s. Plasmids containing inserts of the amplified single copy of *D. immitis* wsp gene sequence were prepared for use as standards in the quantitative PCR. Sample detection was based on the SYBR Green I dye incorporation with the intended PCR products. The specificity of amplification was confirmed by melting curve analysis.

**Results**

After varying conditions for extraction, we could enrich Wolbachia from *D. immitis*. Under a microscope, we found a lot of shiny bacteria-like cells in the preparation. The coccobacilli, with pleomorphic shapes of Wolbachia, were most arranged in single and double cells (Figure 2).

Protein profiles of Wolbachia enriched extracts on the Coomassie blue stained-SDS-PAGE gel showed that using higher NP-40 concentration provided higher protein concentration in pellet, but lower protein concentration in supernatant (Figure 3). The homogenization buffer which provided the highest ration of Wolbachia protein/total parasite extract protein was 0.85% NaCl + 0.08% NP-40. However, the Wolbachia protein concentration was dramatically decreased when using 0.85% NaCl + 0.10% NP-40 as the homogenization buffer.
The *Wolbachia* quantity and the parasite contamination were quantified by real-time PCR. The higher concentration of NP-40 could increase the yield of bacterial isolation and decrease the host parasite contaminations. The homogenization buffer provided the highest ration of \( \text{wsp/gst} \) was 0.85\% NaCl + 0.08\% NP-40. However, the \( \text{wsp/gst} \) ratio was dramatically decreased by using 0.85\% NaCl + 0.10\% NP-40 as the homogenization buffer.

**Figure 2.** Wet-mount preparation of *Wolbachia* pellet from *Dirofilaria immitis* adult worms (A) Total parasite extract; (B) *Wolbachia* cells.

**Figure 3.** Protein analysis of *Wolbachia* purification from *Dirofilaria immitis* by various concentrations of NP-40 (0.04-0.10\%) by SDS-PAGE. M: Markers; E: parasite extracts; and P: *Wolbachia* pellets.
Discussion

Wolbachia have been implicated as novel drug targets for treatment of human filarial parasitic infections, and as the organism triggering the immunopathogenesis.(19, 20) Potential drug targets as well as candidate immunomodulatory/proinflammatory/antigenic molecules of the bacteria can be identified by analyzing the filarial Wolbachia genome.(21) However, a selection process is still needed to validate the protein expression levels. The nature of Wolbachia residing in host vacuole makes limitation on obtaining these purified strictly intracellular bacteria with host free materials, and has hampered progress in the detailed study of their biology. To harvest Wolbachia protein materials for analysis, the isolation protocol for filarial nematode Wolbachia was optimized.

The purification method of rickettsia bacteria from their host cells depends on the isopycnic density gradient centrifugation.(22) However, this technique does not effectively purify Wolbachia from the host materials.(15) The modified method using 0.1-1% Lubrol detergent and 3-μm-pore-size membrane filtration membranes is reported to isolate Wolbachia bacteria from Drosophila eggs.(15) The detergent removes host vacuolar materials from Wolbachia but it also generates the membrane aggregates from the host materials.(15) Therefore, the host material contaminations are found in the Wolbachia fractions after the filtration. The major contaminations are mitrochondria. The further modification of the Wolbachia isolation method is reported based on the purification of Drosophila mitochondria.(16) In this study, they used fractioned centrifugation method by using 0.85% NaCl supplemented with 0.001% of NP-40 detergent in the homogenization buffer. The lower concentration of detergent decreases Wolbachia aggregation with the worm tissue fragments, but results in the lower yield of Wolbachia.(15) Lubrol and NP-40 are non-ionic detergents. Lubrol detergent causes the DNA degradation, while NP-40 is the most common

![Figure 4. The relative concentration of wsp (Wolbachia gene) and gst (Dirofilaria immitis gene) copies in Wolbachia pellets extracted from D. immitis by using various concentrations of NP-40 quantified by real-time PCR](image-url)
used detergent in cell lysis buffer. (17) In this study, we therefore modified the fractioned centrifugation method by using 0.85% NaCl supplemented with varying concentration of NP-40 as the homogenization buffer to find the appropriate concentration of NP-40 which can provide the highest yield but the lowest host material contamination.

From this study, the modified protocol led to massive enrichment of Wolbachia proteins from the total parasite extracts. The use of 0.85% NaCl + 0.08% NP-40 as the homogenization buffer was found to facilitate the proteomic studies. However, Wolbachia bacteria broke up when 0.85% NaCl + 0.1% NP-40 is used as homogenization buffer.

However, like purification of other strictly intracellular bacteria, there are co-purified host proteins, which have nature major in cytoplasm of eukaryotic cells. (23, 24) During the procedures of the Wolbachia isolation, we detected, for example, paramyosin, heat-shock protein, and beta-tubulin of D. immitis by using quantitative real time PCR (data not shown). The proteins may be closely associated with that of the bacteria by the fact that some of them can function in maintaining the cytoskeleton architecture. (25, 26) Therefore, they are difficult to trade-off during purification. The Wolbachia prepared from this isolation method would be an optimal starting material for further extensive purification such as an antibody-affinity chromatography.

Acknowledgments

We are thankful for the support from the National Research Council of Thailand. VS is also financially supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (PHD/0169/2543), and Graduate Thesis Grant from Graduate School, Chulalongkorn University. Moreover, we would like to thank Ms. Chantima Porksakorn, Ms. Alisa Junpee, as well as the staff of the Lymphatic Filariasis Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University for their technical assistance in laboratory works.

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