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Detection of *Wolbachia* endobacteria in *Culex quinquefasciatus* by Gimenez staining and confirmation by PCR

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ABSTRACT

Background & objectives: Wolbachia are common intracellular bacteria that are found in arthropods and nematodes. These endosymbionts are transmitted vertically through host eggs and alter host biology in diverse ways, including the induction of reproductive manipulations, such as feminization, parthenogenesis, male killing and sperm-egg incompatibility. Since they can also move horizontally across species boundaries, *Wolbachia* is gaining importance in recent days as it could be used as a biological control agent to control vector mosquitoes or for paratransgenic approaches. However, the study of *Wolbachia* requires sophisticated techniques such as PCR and cell culture facilities which cannot be affordable for many laboratories where the diseases transmitted by arthropod vectors are common. Hence, it would be beneficial to develop a simple method to detect the presence of *Wolbachia* in arthropods.

Method: In this study, we described a method of staining *Wolbachia* endobacteria, present in the reproductive tissues of mosquitoes. The reliability of this method was compared with Gram staining and PCR based detection.

Results: The microscopic observation of the Gimenez stained smear prepared from the teased ovary of wild caught and *Wolbachia* (+) *Cx. quinquefasciatus* revealed the presence of pink coloured pleomorphic cells of *Wolbachia* ranging from cocci, comma shaped cells to bacillus and chain forms. The ovaries of *Wolbachia* (–) cured mosquito did not show any cell. Although Gram's staining is a reliable differential staining for the other bacteria, the bacterial cells in the smears from the ovaries of wild caught mosquitoes did not take the stain properly and the cells were not clearly visible. The PCR amplified product from the pooled remains of wild caught and *Wolbachia* (+) *Cx. quinquefasciatus* showed clear banding, whereas, no banding was observed for the negative control (distilled water) and *Wolbachia* (–) *Cx. quinquefasciatus*.

Interpretation & conclusion: The Gimenez staining technique applied, could be used to detect the members of the endobacteria *Wolbachia* easily, even in a simple laboratory without any special facilities or even in the field condition and for handling large number of samples in a shorter duration.

Key words *Culex quinquefasciatus*, Gimenez staining, PCR, *Wolbachia* endobacteria

INTRODUCTION

The members of the genus *Wolbachia* are cytoplasmically inherited, intracellular bacteria beloning to the *Rickettsiaeceae* family[1,2]. These bacteria are extremely wide spread; found in the reproductive tissues of over 80% of insect species[3], can cause a number of reproductive alterations in the host, including cytoplasmic incompatibility (CI), parthenogenesis induction (PI) and feminization of genetic males[4]. These intracellular bacteria are known to alter the early development and mitotic processes in the host[5] and have been proposed to be included in applied strategies to control insect vector populations[6]. *Wolbachia* is also common in filarial nematodes where its removal from the female filariae by antibiotic treatments caused permanent sterilization[7] or resulting in embryo degeneration in the filarial worms *Brugia pahangi* and *Dirofilaria immitis*[8] and in worm sterility in *Onchocerca volvulus*[9]. The use of antibiotics targeting *Wolbachia* was suggested to cure patients infected with filarial worms. In addition, *Wolbachia*'s intimate relationship with their hosts and ability to spread in their host population make them good candidate for paratransgenic approaches[10].

However, *Wolbachia*'s obligate intracellular life style renders these approaches difficult[11]. The study of *Wolbachia* requires PCR-based methodologies[12,13] or cell culture facilities[14] which are very expensive and also require special expertise. Many such facilities are not affordable in tropical countries where the diseases transmitted by mosquitoes are prevalent. In this study, we describe a method to detect the presence of *Wolbachia* in *Culex quinquefasciatus*, the mosquito that act as vector of lymphatic filariasis, using Gimenez staining and com-

pared with Gram's staining and confirmed by PCR assay. The experiments were carried out in triplicate. The staining method we used was originally developed by Gimenez, in 1964[15] to identify the bacteria belonging to the genus, *Rikketsiae* in infected yolk sacs. The primary stain for this technique is carbol fuchsin, and the secondary stain is malachite green while the molecular basis of Gimenez staining remains to be determined, it is known that the basic dye carbol fuchsin is retained by the acid fast bacteria, typically mycobacteria, which have a complex envelope composed of glycolipids and glycopeptido lipids[16]. Gimenez stain yields a clear cut staining of rikketsiae, which appears as pink/red whereas the underlying tissue is blue/green.

MATERIAL & METHODS

Rearing of mosquitoes

The immature stages of *Cx. quinquefasciatus* were collected from the stagnant polluted water of drains in Madurai Corporation area of Tamil Nadu, India. The larvae were pooled together and reared at a density of 300 larvae/litre. Ground biscuit crumbs (Dog biscuits with less oil) and baker's yeast (3:2 ratio) were the source of the feed for these larvae. The pupae after emergence were collected and transferred into 30 cm^3 mosquito cages. The adults were provided with cotton soaked on 10% sucrose solutions which were changed at regular time intervals. All the experiments were carried out inside the insectaries which were maintained at 26°C, 50% RH with a 16:8 L:D photoperiod. The females were separated out and the ovaries were dissected. Each ovary was transferred to a clean glass slide containing a drop of saline water, mechanically teased with sterile needle, dried and fixed with heat. The remaining part of the ovary and carcass of the adult mosquito were used for PCR identifications.

Tetracycline treatment to remove Wolbachia infection

Wolbachia from mosquitoes were cured by feeding adults with 10% sucrose supplemented with 1 mg/ml tetracycline (pH 7) following the procedure of Dobson and Rattanadechakul[17]. Tetracycline from the sucrose was removed after 1 week. Mosquitoes were provided two consecutive blood feedings to collect eggs[18]. The process was repeated at least for 2 generations. At generation 3, the removal of *Wolbachia* from mosquito was confirmed by testing 10 individual ovaries.

Gimenez staining

The fixed smears of (either wild caught or *Wolbachia* cured) were flooded with freshly filtered carbol fuchsin solution prepared from 2 ml of stock solution of basic fuchsin in 5 ml of phosphate buffer for 10 sec. The smear was then rinsed in tap water, and incubated in malachite green solution for 9 sec. The smear was then rinsed again in tap water and was air-dried[15]. The slide was examined for the presence of *Wolbachia* species under a compound microscope (Zeiss) attached with photo-micrographic unit at a magnification of ×1000.

Gram staining

Gram staining of the smears was performed as described previously[19]. Briefly, the slides with the smear of teased ovary (either wild caught or *Wolbachia* cured) were heat fixed and then the primary stain crystal violet was added and incubated for 1 min. After washing, Gram's iodine was added on the smear and kept for 30 sec. After decanting the Gram's iodine, the smear was washed with the decolourizer—ethanol. Then the secondary stain, safranin was added, and incubated for 1 min. The smear was then washed with tap water for a maximum of 5 sec and was examined under a compound microscope for the presence of *Wolbachia*.

DNA extraction and PCR

The total DNA was separately isolated from the three sets of *Cx. quinquefasciatus*, the first set contains five pools of wild caught mosquitoes (the presence of *Wolbachia* was confirmed by staining), the second set contains one pool of *Wolbachia* (+) mosquitoes and the third set contains two pools of *Wolbachia* (–) cured mosquitoes. Totally eight pools were prepared (5 pools of testing wild caught mosquitoes +1 pool of Wol (+) and 2 pools of Wol (–) mosquitoes) each pool consisted of two mosquitoes. The remaing carcasses without ovaries were processed for DNA isolation. For DNA extraction "DNA Extraction Solution" kit (Genei, Bangalore, India) was used and the user recommended protocols were followed. Individual reaction mixtures prepared from 25 µl contained 1 µl of forward and reverse primers, with 2.5 µl of buffer containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 0.1% Triton x 100 supplemented with 1.5 mM $MgCl_2$ (Promega), 200 µl of each dNTPs (GeneCraft), 1 µl of Taq DNA polymerase (Promega) and 3 µl of gDNA. The primers used in the study were 16S *Wolb* F (5' TTG TAG CCT GCT ATG GTA TAA CT 3') and 16S *Wolb R* (5' GAA TAG GTA TGA TTT TCA TGT 3') 1µl (O'Neill *et al*[1]). The conditions for amplifications were: one cycle of 2 min at 94°C, 35 cycles of 30 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C and final extension at 72°C for 10 min. A negative control for the PCR assay (sterile distilled water instead of DNA extract in the reaction mix-

ture) was included in each run. Finally, the PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.51 g/ml).

RESULTS & DISCUSSION

The microscopic observation of the Gimenez stained smear prepared from the teased ovary of wild caught (n=10) and *Wolbachia* (+) (n=2) *Cx. quinquefasciatus* revealed the presence of pink coloured pleomorphic cells of *Wolbachia* ranging from cocci, comma shaped cells to bacillus and chain forms (Fig. 1a). The Gimenez stained slides prepared from the ovaries of *Wolbachia* (–) cured mosquito (total 4) did not show any cell (Fig. 2). Although Gram's staining is a reliable differential staining for the other bacteria, the bacterial cells in the smears from the ovaries of wild caught, mosquitoes did not take the stain properly and the cells were not clearly visible (Fig. 1b).

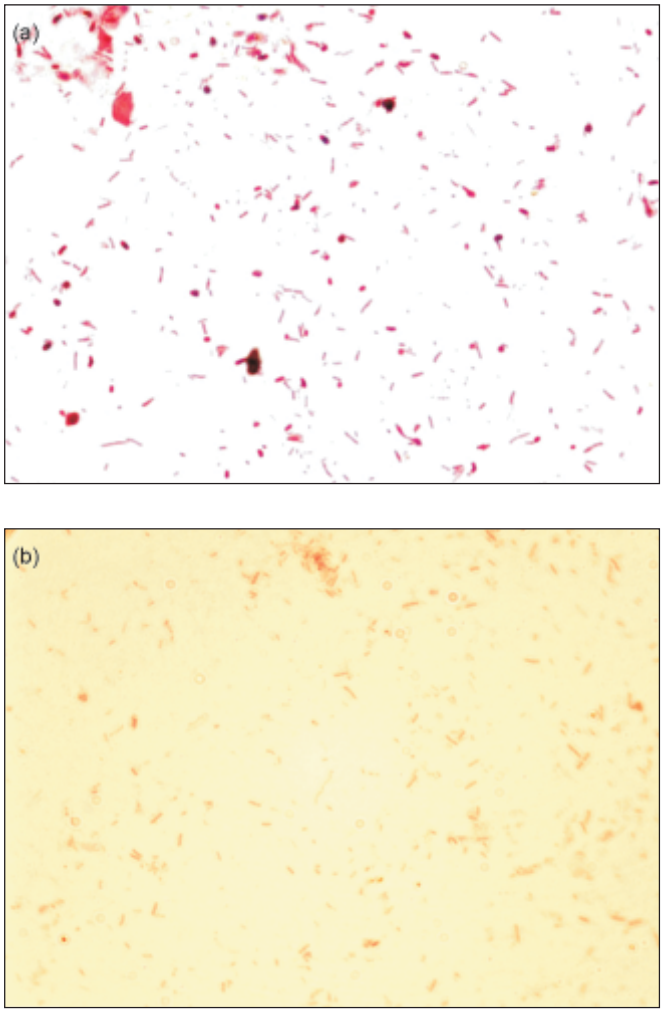

Fig. 1: Slides stained by Gimenez staining: (a) Gram staining; and (b) The typical pink coloured pleomorphic cells of *Wolbachia* were clearly visible in slides stained with Gimenez staining. The slides stained by Gram's method gave poor visibility of cells and cells are not properly stained.

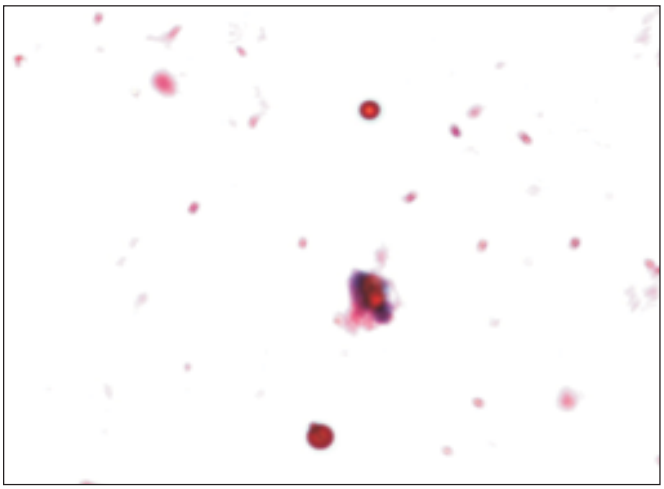

Fig. 2: The slide of teased ovary from *Wolbachia* (–) mosquito did not show any bacterial cells by Gimenez staining.

The PCR amplified product from the pooled remains of wild caught female *Cx. quinquefasciatus* along with remains of the ovary left over after making smears for Gimenez and Gram's staining, showed clear banding at 900 bp region in the lanes L1 to L5 since the product size of used Wp 16S primer is 890 bp which generally appear at par with marker having size of 900 bp. The positive control used, *Wolbachia* (+) gave band at L6, exactly as the wild caught mosquitoes. No banding was observed for the negative control (distilled water) and *Wolbachia* (–) mosquitoes at L7, L8 and L9 respectively (Fig. 3).

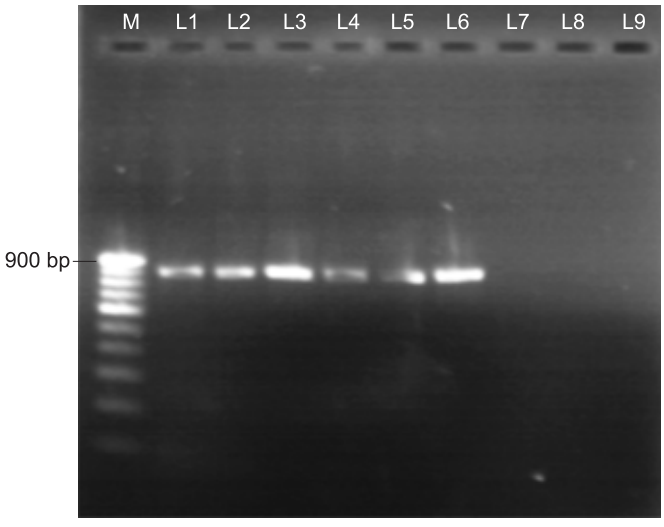

Fig. 3: The PCR amplified products from the *Cx. quinquefasciatus* confirms the presence of *Wolbachia*. M–Molecular weight marker; lane L1 to L5–Samples already confirmed for *Wolbachia* by Gimenez staining; L6–Positive control; L7–Negative control; and L8 & L9–Mosquito samples cured of *Wolbachia* infection.

The results show that the staining technique applied, Gimenez could be used to detect the members of the endobacteria *Wolbachia*, even in a simple laboratory without special facilities and also even in the field condition. Although Gram's staining showed the presence of cells, they appeared very poor and cannot be used as a technique to detect the presence of *Wolbachia* bacteria. The mosquito samples that gave positive for *Wolbachia* in Gimenez staining were confirmed to be positive for *Wolbachia* by PCR. Although, Gimenez stain has been used to stain the various rickettsial bacteria such as Bartonella, Coxiella, etc[20], it has not been reported to be used for the detection of *Wolbachia* from mosquito ovaries. Application of this staining to detect *Wolbachia* was used during the study period and was found to be more efficient than the Gram staining (Fig. 2). The Gimenez staining could be a potential tool in the detection of *Wolbachia*, especially for the initial screening of large number of samples to determine the diversity in the natural environment.

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