ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 08, pp.35771-35773, August, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

IN VITRO ESTABLISHMENT OF *WOLBACHIA* INFECTION IN THE OVARIAN CELL LINES OF (DZNU Bm-12) SILKWORM, *Bombyx mori L*

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ARTICLE INFO

Article History:
Received 21st May, 2016
Received in revised form
20th June, 2016
Accepted 25th July, 2016
Published online 20th August, 2016

Key words:

Wolbachia,
Silkworm cell line (DZNU Bm-12),
PCR, RML-12 *Aedes albopictus*.

ABSTRACT

The *Wolbachia* is a maternally inherited, gram negative, obligate, intracellular proteobacteria which is found in number of invertebrates, except in the silkworm. To understand the *Wolbachia*-hosts interaction and molecular mechanism by which *Wolbachia* would interfere with viral replication,This symbiotic *Wolbachia* bacterium was isolated from RML-12 *Aedes albopictus* cells and was transfected into silkworm ovarian cell lines (DZNU Bm-12) maintained under *in vitro* standard conditions. The infection level was determined by PCR assay. None of the 1 to 40th passage showed infection in the silkworm. However, the techniques helped in understanding the incompatibles involved in non-infection of *Wolbachia* in silkworm. This study has opened out the scope for further assessment on the compatible factors.

Citation: Kiran, H.R., Kishore V.P., Dr. Puttaraju, H.P. and Ashwini, S. 2016. "*In vitro* establishment of *Wolbachia* infection in the ovarian cell lines of (DZNU Bm-12) silkworm, *Bombyx mori L*", *International Journal of Current Research*, 8, (08), 35771-35773.

INTRODUCTION

The Silkworm *(Bombyx mori*) is an economically important agricultural insect and it is a producer of silk. It is susceptible to viral diseases, but it is not infected with *Wolbachia*. *Wolbachia is* a group of obligate, intracellular, α-proteobacterial endosymbiont that infects a wide variety of arthropods. It induces a broad range of reproductive phenotypes to ensure its persistence in the host population (Werren *et al.*, 2008). As *Wolbachia* is an endosymbiont, it was not possible to culture it in *in-vivo* conditions. However, recently it was successfully cultured in *in-vitro* conditions in the cell lines of several insects and mammals. Through these cultures various interesting insights has been observed. Lemaitre *et al.* (2007) proved that when *Wolbachia* was cultured in *Drosophilla* cell lines, the Immune Mediated Disease pathway was activated by gram negative bacteria that induces the synthesis of potent antimicrobial peptides (AMPs) such as attacin, cecropin, drosocin, and diptericin. In other such studies, Hedges *et al*., (2008) and Teixeira *et al.,* (2008) showed that *Wolbachia* provide antiviral protection from the RNA viruses. Two different *Wolbachia* strains was showed to remain in the continuous cell line as a carrier culture in Asian tiger mosquito, *Aedesalbopictus* and being extensively used in assaying insect immunology by Hiroaki Noda *et al*., 2002). Similarly, small brown plant hopper, *Laodelphaxstriatellus*, infected by *Wolbachia*, an endosymbiont was cultured and maintained in insect and mammalian cell lines in *in vitro* conditions. A mouse cell line L929 was tested for *Wolbachia* cultivation. (Hiroaki Noda *et al*., 2002). These studies have indicated that *Wolbachia* plays a major role in activating the innate immune responses in the cell lines and induces pathogenesis. Based on the earlier studies, it is evident that insects infected with this bacteria as endosymbiont are protected from pathogenic viruses. It has proved to be a major contributing factor for antiviral defense in *Drosophilla melanogaster* and in mosquitoes. The bacterial endosymbiont *Wolbachia pipientis* was proved to increase the host resistance to viral infection in native Drosophila host (Hedges, 2008). *Wolbachia* was established in cell cultures of silkworm, its differential expression of host genes which was involved in immune response of silkworm cells associated with *Wolbachia* were identified (Kageyama 2008; Nakamura 2011). This has lead to understand the vital potential of *Wolbachia* to influence

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the outcome of host pathogen interactions in the different host background. The Transfected Silkworm cell line was previously developed in the laboratory by Kageyama.*et al*., (2008). From the previous studies in *Drosophila* species, it is clear that the insects with the natural infection of *Wolbachia* showed a strong immune response when compared to experimental induction and some of the strains showed this response under different host background. Since vertical transmission is not favoured in silkworm, transfection of *Wolbachia* does not persist in silkworm host. Keeping in view of the above, there is a need to understand the genes of *Wolbachia* involved in host gene up-regulation which directly participate in the viral inhibition. Therefore, to understand the *Wolbachia*-hosts interaction and molecular mechanism by which *Wolbachia* would interfere with viral replication, the present study was taken up to establish *Wolbachia* in the silkworm ovarian cell line. The present study was also an attempt to develop a transgenic silkworm which is resistant to the viral infection.

MATERIALS AND METHODS

Maintenance and culturing of cell lines

A new continuous silkworm ovarian cell line as DZNU-Bm12 (Adherent) were maintained in 25cm^3 of tissue culture flask (T-flask) with culture medium which was used is the TNM-FH grace supplemented with 10% foetal bovine serum (FBS) and cultures condition at 25±1^{0}C temperature was maintained in the lab. The culture medium was replenished half of the medium once a week.

Wolbachia Purification and Infection

The *Wolbachia* was purified from RML 12 *Aedes albopictus* cell lines which was grown in 25 cm^{2}of plastic tissue culture flasks with 90% confluence. Accordingly, further purification procedure of centrifugation and filtration techniques was followed according to the method of Rasgon *et al*., (2006). The Purified Wolbachia bacteria were introduced into the Silkworm ovarian cell lines (DZNU Bm-12) which was grown in TNM-FH culture medium (Hi media) and supplemented with 5% Foetal bovine serum (FBS) to 80-90% confluence in the 25 cm^3 of plastic tissue culture flask. The infections were carried out by innoculum whose strength has been standardized (~10-15 micro liters) and were maintained under *in vitro* condition.

DNA extraction

DNA was extracted from *Wolbachia* inoculated DZNU Bm-12 cells by using kit method (DNA easy Blood and tissue kit, Qiagen) and quantity of the DNA was confirmed by using 2% agarose gel electrophoresis. The gel images were taken from Bio-Rad gel documentation system and the purity and concentration of DNA were determined by measuring the OD at 260 - 280nm, then *Wolbachia* confirmation was estimated by PCR assay using *wsp* general primer.

PCR Assay

A polymerase chain reaction assay was carried out based on the amplification of *Wolbachia* specific *wsp* general gene primers: *wsp*-81F (5'-TGGTC CAATAAGT GATGAAGAA AC -3'), *wsp*-691R (5'-AAAAATTAAACGCTACTCCA-3') (Zhou *et al.,* 1998; Braig *et al*., 1998), which were used to determine the infection of *Wolbachia*. Amplification was done by PCR thermocycler (Eppendorf)) and 1 µl of DNA samples in a reaction volume of 25 µl. The reaction mixture contained 10 µl 10X PCR buffer, 3 µl 25 mM $MgCl_2$, 1.25 µl dNTPs (10 mM each), 1 µl 20 pmoles of both forward and reverse primers and 1 Unit of Taq DNA polymerase (5' prime Effendrof) and the final volume was made up to 25µl with nuclease free water. A negative control for the PCR assay (sterile distilled water instead of DNA in the reaction mixture) and PCR samples were run under the following cyclic conditions: 95 °C for 3 min, followed by 35 cycles 95 °C for 1 min, 55 °C for 1min and 72 °C for 2 min, then final extension 72 °C for 10 min. The analysis of the PCR products was conducted by gel electrophoresis. 10 µl of the PCR product was mixed with Green View^{TM} DNA loading buffer (Chromous Biotech^{TM}, Bangalore) and was loaded into 2% agarose gel and the amplicons were documented by gel documentation unit (Alpha Imager $^{(R)}$ EP, San Leandro, USA). The presence/ absence of the band indicated the prevalence and diversity of *Wolbachia* infections.

RESULT AND DISCUSSION

The endosymbiont *Wolbachia* present in a wide range of insect and plays important role in host reproduction alteration and fight against the viral diseases. *Wolbachia* is known to survive for a week in a cell-free media (Rasgon *et al.,* 2006). Screening for *Wolbachia* was made to detect *Wolbachia* in the silkworm and mosquitoes through diagnostic PCR technique. *Wolbachia* infection was successful in mosquitoes and unsuccessful in Silkworm. This initial result is in Congruence with earlier reports of absence for *Wolbachia* infection in silkworm (Puttaraju, *et al*., 2002 and Prakash, *et al*., 2007). Therefore live *Wolbachia* was purified from RML *12 Aedes albopictus* cells which is maintained at low temperature (Below 4 ^{0}C) and inoculated into the silkworm cell line (DZNU Bm-12), within 8 hrs of isolation as previously reported by Noda et al., 2011. Based on this study the presence of *Wolbachia* was not found in silkworm cell line even in the 1 to 40th passage using PCR Technique and *wsp* specific primers (Zhou *et al*., 1998). However *Wolbachia* infected RML-12 *Aedes albopictus* cells were maintained in the lab at standard condition as a positive control and fragment amplification seen in *Wolbachia* infected RML-12 *Aedes albopictus* cells were amplified around 590bp.

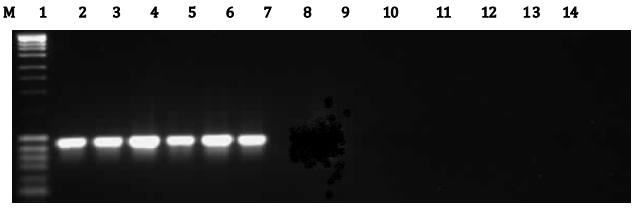

Figure 1. PCR screening for the absence of *Wolbachia* in DZNU Bm-12 cells and the Presence of *Wolbachia in* RML 12 *Aedes albopictus* cells using general *wsp*specific primer .M-Marker, Lane 01-06: RML 12 *Aedes albopictus* cells of 5th,10th,15th,20th,25th and 30th passage infected with *Wolbachia* (Positive control), Lane 07-14: DZNU Bm-12 cells of 5th ,10th,15th,20th,25th,30th ,35th and 40th passage inoculated with *Wolbachia*

Comparison between *Wolbachia* infected and uninfected cell lines reaching delayed confluence

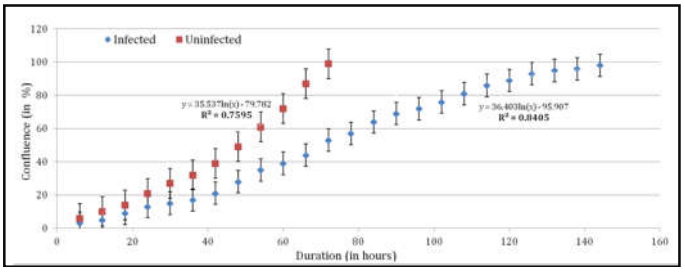

Figure 2. Confluence between *Wolbachia* infected and uninfected cell lines

The *Wolbachia* inoculated Silkworm cell lines, passages were regularly estimated for bacterial densities after reaching confluence and it was observed that the cell lines inoculated with *Wolbachia* reached delayed confluence. The inoculum's strength was standardized by *Wolbachia* load in each passage. Proliferation was calculated based on the cell multiplication and cell count under high phase contrast in inverted microscope. The cell colony counter was used to monitor the differential proliferation of *Wolbachia.* However, there is an interesting two fold retardation in the proliferation of cells. The *Wolbachia* infection delays confluence of cells by over 48 hours. Noda et.al (2002) is reported that WcauA, Wkue and Wstr strain are isolated from plather can easily infected mosquitoes cells. In the present study was Wolbachia infection was found to be negative.

Summary / Conclusion

1 to 40th cell passages were done to identify the infection of *Wolbachia* in silkworm ovarian cell lines. None of the passages showed the infection. However Noda *et al.* (2008) transfection of *Wolbachia* into *Bombyx mori* have Shown the somatic infection of *Wolbachia* but no stable infection. The non-infection of *Wolbachia* in silkworm ovarian cells may be due to incompatibility factors. These has enough scope to study in the future factor for non-infection of *Wolbachia* in the silkworm.

Acknowledgement

We are grateful to funding agency DST-SERB, department of Science and Technology, Science and Engineering Research Board, New Delhi.

Conflicts of Interest : No conflict of interest.

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