ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 11, pp. 14177-14180, November, 2016

International Journal of Recent Scientific Research

Research Article

ANALYSIS OF HUMAN HEPATOCYTE CELL LINES FOR IN VITRO CULTURE AND ESTABLISHMENT OF *WOLBACHIA*

Kishore. V. P.[1]* and Puttaraju. H. P.[2]

[1,2]Department of Biological Sciences/Life Science Bangalore University, Bangalore, Karnataka, India

ARTICLE INFO

Article History:

Received 15th August, 2016
Received in revised form 25th September, 2016
Accepted 28th October, 2016
Published online 28th November, 2016

Key Words:

Wolbachia, Human hepatocyte cells, Infection, Establishment, Effect, Polymerase Chain Reaction (PCR) Assay.

ABSTRACT

Wolbachia, an endosymbiont has been reported to act on the immune responses of its host and hinder other pathogenic microbes. Keeping this in view, it was necessary to screen and characterize *Wolbachia*. Hence it was essential to know, can *Wolbachia* infection be established in Human cell lines to study the effect of *Wolbachia* in uninfected Human cell host or to investigate *Wolbachia* – host cell interactions. Thus, in this study, we isolated *Wolbachia* and inoculated into the cultures of MRA 975 Human hepatocyte cell lines to examine the effect of *Wolbachia* in Human hepatocyte cells. *Wolbachia* infection of cells were assessed by Polymerase Chain Reaction (PCR) Assay, which indicated that MRA 975 Human hepatocyte cells was not infected with *Wolbachia*. So, our study suggests that *Wolbachia* cannot be infected, cultivated and established in MRA 975 Human hepatocyte cells to study the effect of *Wolbachia* or to investigate *Wolbachia* – host cell interactions.

INTRODUCTION

Wolbachia is maternally inherited gram negative, obligate intracellular endosymbiotic, alpha proteo bacteria found widely in arthropods and filarial nematodes but not in mammals.

Wolbachia being an endosymbiont, can live only in the cytoplasm of their host's cells. It was impossible to culture *Wolbachia* in in vivo conditions. *Wolbachia* require cells for in vitro cultivation and have been cultivated in established cell lines. *Wolbachia* have been cultured in cell lines of insects and mammals. The first example of a mosquito cell line persistently infected with *Wolbachia* (S. L. O'Neill *et al*, 1997; Sinkins S. P. and Godfray H. C. J. 2004) is the Aa23 cell line (S. L. O' Neill *et al*, 1997). *Wolbachia* strains which can be maintained in *Anopheles gambiae* cell lines (Jason. L. Rasgon *et al*, 2006; Conor. J. McMeniman *et al*, 2008), suggest that there is no *Wolbachia* infection preventing mechanism in *Anopheles* cells. In vitro cultivation of different strains of *Wolbachia* was achieved using several insect cell lines (Stephen. L. Dobson *et al*, 2002). Through these cultures numerous interesting insights have been observed. The gram negative bacteria activate the Immune Mediated Disease pathway inducing the synthesis of potent antimicrobial peptides (AMPs) like cecropin, attacin, drosocin and diptericin when *Wolbachia* were cultured in *Drosophila* cell lines (Bruno Lemaitre and Jules Hoffmann, 2007). In other such studies it has been shown *Wolbachia* provide antiviral protection from RNA viruses (Lauren. M. Hedges *et al*, 2008; Luis Teixeira *et al*, 2008). Antiviral protection from RNA viruses provided by *Wolbachia* has been shown in other studies (Lauren. M. Hedges *et al*, 2008; Luis Teixeira *et al*, 2008). Two different *Wolbachia* strains, have remained in the continuous cell line as a carrier culture in Asian tiger mosquito, *Aedes albopictus* (Hiroaki Noda *et al*, 2002) and are being widely used in examining insect immunology. Likewise, *Wolbachia* which infect the small brown planthopper, *Laodelphax striatellus*, were cultured and maintained in the cell lines of insect and mammals. *Wolbachia* cultivation has also been tested on L929 mouse cell line (Hiroaki Noda *et al*, 2002). Since these studies showed that *Wolbachia* act on the immune responses of its host and hinder other pathogenic microbes, it will be necessary to screen and characterize them. Very little reported information is available on the interaction of *Wolbachia* in mammals and mammalian cell lines. Hence it is essential to know, can *Wolbachia* infection be established in Human hepatocyte cells in order to study the effect of *Wolbachia* in uninfected Human cell host background.

In this study, we isolated *Wolbachia* from RML 12 *Aedes albopictus* Mosquito cells harboring *Wolbachia* and inoculated *Wolbachia* into the cultures of MRA 975 Human hepatocyte cell lines to examine the ability of the cell lines to harbor *Wolbachia* infection. Examination of the ability of the cell lines

*Corresponding author: **Kishore. V. P.***
Department of Biological Sciences/Life Science Bangalore University, Bangalore, Karnataka, India

to harbor *Wolbachia* infection is essential to screen and characterize the effect of *Wolbachia* in Human hepatocyte cells or to investigate *Wolbachia* – host cell interactions. Human hepatocyte cells are used in this study, as they are used in malarial vaccine studies, antimalarial testing, liver stage development of *Plasmodium falciparum* and *P. vivax*. In the present study, we report on the possibility of infection, cultivation and establishment of *Wolbachia* in MRA 975 Human hepatocyte cells.

MATERIALS AND METHODS

Cell lines and its maintenance

Cell lines used in this study

a) MRA 975 Human Hepatocyte cell line
MRA 975 Human Hepatocyte cell line was procured from BEI Resources, American Type Culture Collection (ATCC), USA.

MRA 975 Human Hepatocyte cells were maintained in a T-25 flask containing HC-04 Complete Culture Medium (CCM) which contains equal volume of Minimum Essential Medium (MEM) and F-12 Nutrient mix supplemented with 10% Fetal Bovine Serum (FBS), 15mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1.5g/L sodium bicarbonate and 2.5mM L-glutamine (GIBCO, INVITROGEN, USA) in CO_2 incubator at 37^0C with 5% CO_2. Media was changed at 12-16 hours post seeding. Cells were fed at least every 48 hours and were harvested at 80% confluency.

b) RML 12 *Aedes albopictus* Mosquito cell line harboring wMel Pop strain *Wolbachia* and RML 12 *Aedes albopictus* Mosquito cell line without *Wolbachia*

RML 12 *Aedes albopictus* cell lines harboring *Wolbachia* and *Wolbachia* uninfected RML 12 *Aedes albopictus* cell lines were procured from Prof. Scott O'Neill, Professor Monash University, Clayton, Australia.

RML 12 *Aedes albopictus* cell lines were maintained in growth medium consisting of equal volumes (1:1; vol/vol) of Mitsuhashi and Maramorosch (M and M) Insect media, Schneider's Insect Medium supplemented with 10% Heat-Inactivated Fetal Bovine Serum (HIFBS). Media also contained Penicillin (50mg/mL) and Streptomycin (50mg/mL) (GIBCO, INVITROGEN, USA).

RML 12 *Aedes albopictus* cells were grown in 25 cm^2 culture flasks containing 5 mL of medium at 26^0C without CO_2 incubation. Cells were passaged every 3-4 days (when 90-100% confluent) by vigorous shaking (tapping against hand until all of the cells become loose) of the flask and seeding a new flask with 20% of the resuspended cells and 5 mL fresh media.

For the production of large amounts of *Wolbachia*, RML 12 *Aedes albopictus* cells harboring *Wolbachia* were harvested from one ask for every 5 days and was inoculated into three cell culture asks with fresh medium (1:3 split).

Wolbachia isolation

Wolbachia was isolated from RML 12 *Aedes albopictus* cells harboring *Wolbachia* using cell disruption and filtration techniques according to Jason. L. Rasgon *et al*, 2006 protocol mentioned below.

For isolation of *Wolbachia*, RML 12 *Aedes albopictus* cells harboring *Wolbachia* were grown in 50cm^2 culture asks to 90% con uence. Cells were washed using phosphate-buffered saline. Cells were pelleted by centrifugation at 2,500g and 4°C for 10 minutes, the supernatant was removed and the cells were resuspended in 10mL media in a 50mL conical tube. Cells were vortexed for 5 minutes with about 100 sterile 3mm glass beads to lyse cells. The lysate was centrifuged at 2,500g at 4°C for 10 minutes to pellet huge cellular debris. The supernatant was decanted, passed through a 5m Millex syringe lter (MILLIPORE, Billerica, MA), and centrifuged at 18,400g at 4°C for 5 minutes on a 250mM sucrose cushion to pellet *Wolbachia*. The *Wolbachia* pellet was resuspended in 1mL Schneider's media with 10% Fetal Bovine Serum and passed through 2.7m syringe lter (WHATMAN, Florham Park, NJ) to take away residual cellular debris (Jason. L. Rasgon *et al*, 2006).

Wolbachia isolated from *Wolbachia* harboring RML 12 *Aedes albopictus* cells was assessed by Polymerase Chain Reaction (PCR) Assay using *Wolbachia* specific *Wolbachia* surface protein (wsp) gene primer.

Wolbachia inoculation

Wolbachia isolated from *Wolbachia* harboring RML 12 *Aedes albopictus* cells was inoculated into MRA 975 Human Hepatocyte cells for infection using the below mentioned protocol.

MRA 975 Human Hepatocyte Cell monolayer was grown in 25-cm^2T ask to 80% con uence. *Wolbachia* suspension (500µL) was layered onto cells and cultured as described.

Wolbachia infection of cells were assessed by Polymerase Chain Reaction (PCR) Assay after every 5 cell passages post infection and throughout the experiment until 50 passages along with *Wolbachia* infected RML 12 *Aedes albopictus* cells which was used as positive control and *Wolbachia* uninoculated MRA 975 Human hepatocyte cells and RML 12 *Aedes albopictus* cells which were used as negative control.

DNA Extraction

By using DN easy kit (QIAGEN, Valencia, CA) total DNA from cells was extracted according to the manufacturer's suggested protocol mentioned below.

Cells were placed in a Ultra Violet (UV) - crosslinked 1.5mL tube. 180µL Buffer ATL and 20µL Proteinase K were added to UV-crosslinked 1.5mL tube and vortexed. The tube was placed in the 55^0C incubator for 3 hours or overnight. Then the tube was removed from incubator, vortexed, 200µL Buffer AL was added and vortexed. The tube was placed in heat block at 70^oC for 10 minutes. 200µL 100% Ethanol was added and entire volume was transferred onto spin column. The spin column was centrifuged at 8000rpm for 1 minute; flow-through was thrown out. 500µL Buffer AW1 was added and centrifuged at 8000rpm for 1 minute; flow-through was thrown out. 500µL Buffer AW2 was added and centrifuged at 13000rpm for 3 minutes; flow through was thrown out. Spin column was placed on UV-crosslinked 1.5mL tube, 200µL buffer AE was added. It was let to settle for 1 minute, then

centrifuged at 8000rpm for 1 minute. The above step was repeated and then flow-throughs were combined for a total volume of 400µL.

Polymerase Chain Reaction (PCR) Assay

Polymerase Chain Reaction (PCR) Assay was used for detection of *Wolbachia* infection in MRA 975 Human hepatocyte cells using *Wolbachia* specific *Wolbachia* surface protein (wsp) primers - wsp-81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3'), wsp-691R (5'-AAAAATTAAACGCTACTCCA-3') (Henk. R. Braig *et al*, 1998; Weiguo Zhou *et al*, 1998). *Wolbachia* amplicon size should range from 590 to 632bp (Weiguo Zhou *et al*, 1998).
PCR amplifications were performed in 25µL reactions containing 1µL of DNA, 2.5µL 10x reaction buffer, 2.0µL $MgCl_2$ (25mM), 1µL dNTPs (25uM), 0.2µL of Taq polymerase and 18.3µL water.

PCRs were performed using the following cycling conditions: an initial denaturation at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94°C, annealing at 55°C, extension at 72°C for 1 minute and a final extension step at 72 °C for 10 minutes.

Agarose Gel Electrophoresis was carried out for the PCR products using 1% agarose gel stained with ethidium bromide and was visualized with Ultra Violet light to determine the presence of the amplified DNA.

RESULTS

Wolbachia isolation

Wolbachia was isolated from *Wolbachia* harboring RML 12 *Aedes albopictus* Mosquito cells using the protocol mentioned. It resulted in very pure *Wolbachia*.

Isolated *Wolbachia* was con rmed by Polymerase Chain Reaction (PCR) ampli cation of *Wolbachia* speci c *Wolbachia* surface protein (wsp) gene.

Screening of Wolbachia infection in MRA 975 Human Hepatocyte cells

MRA 975 Human hepatocyte cells were tried to infect with *Wolbachia* using the protocol mentioned.

Wolbachia infection of MRA 975 Human hepatocyte cells were assessed by Polymerase Chain Reaction (PCR) Assay using *Wolbachia* specific *Wolbachia* surface protein (wsp) primers after every 5 cell passages post infection and throughout the experiment until 50 passages.

PCR amplification was not observed in MRA 975 Human hepatocyte cells inoculated with *Wolbachia.* PCR amplification was also not observed in *Wolbachia* uninoculated MRA 975 Human hepatocyte cells and RML 12 *Aedes albopictus* cells, which were used as negative control but was observed in *Wolbachia* infected RML 12 *Aedes albopictus* cells, which was used as positive control (Fig 1).

Screening of *Wolbachia* infection in MRA 975 Human hepatocyte cells indicated that *Wolbachia* infection was not observed in MRA 975 Human hepatocyte cells.

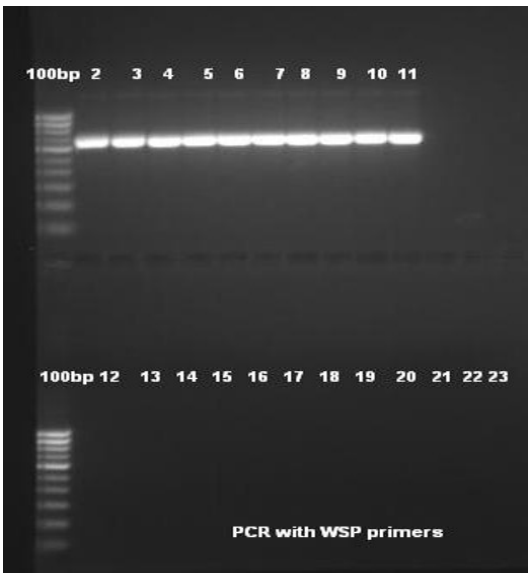

Figure 1 PCR screening of *Wolbachia* using wsp primers

Lane 1: 100bp DNA Marker (Ladder)

Lanes 2-11: PCR amplification of *Wolbachia* harboring RML 12 *Aedes albopictus* Mosquito cells at every 5 cell passages until 50th passage - Positive Control

Lane 12: PCR amplification of RML 12 *Aedes albopictus* Mosquito (*Wolbachia* uninfected) cells - Negative Control
Lane 13: PCR amplification of MRA 975 Human Hepatocyte (*Wolbachia* uninfected) cells - Negative Control
Lanes 14-23: PCR amplification of *Wolbachia* inoculated MRA 975 Human Hepatocyte cells after every 5 cell passages post infection until 50th passage - Sample

DISCUSSION

Wolbachia being an endosymbiont, can live only in the cytoplasm of their host's cells. It was impossible to culture *Wolbachia* in in vivo conditions. *Wolbachia* require cells for in vitro cultivation and have been cultivated in established cell lines.

Mammals have never been found to harbor *Wolbachia* in nature. However, *Wolbachia* have been cultured in vitro in cell lines of insects and mammals. *Wolbachia* which infect the small brown planthopper, *Laodelphax striatellus*, were cultured and maintained in cell lines of insect and mammals. *Wolbachia* cultivation has also been tested on L929 mouse cell line (Hiroaki Noda.*et al*, 2002).

Very little reported information is available on the interaction of *Wolbachia* in mammals and mammalian cell lines. *Wolbachia* has been reported to act on the immune responses of its host and hinder other pathogenic microbes. Keeping these in view, it was necessary to screen and characterize *Wolbachia*. Hence it was essential to know, can *Wolbachia* infection be established in Human hepatocyte cells to study the effect of *Wolbachia* in uninfected Human cell host background or to investigate *Wolbachia* – host cell interactions.

In this study, we inoculated *Wolbachia* into MRA 975 Human hepatocyte cells to examine the ability of the cell lines to harbor *Wolbachia* infection. Examination of the ability of the cell lines to harbor *Wolbachia* infection was essential to screen

and characterize the effect of *Wolbachia* in Human hepatocyte cells or to investigate *Wolbachia* – host cell interactions. Human hepatocyte cells were used in this study, as they are used in malarial vaccine studies, antimalarial testing, *Plasmodium falciparum* and *P. vivax* liver stage development.

Wolbachia infection of cells were assessed by Polymerase Chain Reaction (PCR) Assay after every 5 cell passages post infection and throughout the experiment until 50 passages. From PCR study of *Wolbachia* (Fig 1) it is clearly evident that MRA 975 Human hepatocyte cells was not infected with *Wolbachia*. So, our study suggests that *Wolbachia* cannot be infected, cultivated and established in MRA 975 Human hepatocyte cells and *Wolbachia* with Human hepatocyte cell system could not be potentially used to investigate *Wolbachia* – host cell interactions.

CONCLUSION

In this initial study, attempts were made to infect, cultivate and establish *Wolbachia* in MRA 975 Human hepatocyte cells using the protocol mentioned but were unable to establish infection which may be due to incompatibility of *Wolbachia* with its host. Investigation is required to know the reason for the inability of infection and establishment. Inspite of the efforts made to establish the infection, further detailed investigation using different culture conditions (pH, temperature, media composition, CO_2 %), different methods and different parameters (*Wolbachia* concentrations) etc. is required which may lead to the successful establishment of *Wolbachia* infection.

Acknowledgements

This Research work was funded by University Grants Commission Centre with Potential for Excellence in Particular Area (UGC CPEPA).

We are indebted to Prof. Scott O'Neill, Professor Monash University, Clayton, Australia for kindly providing *Wolbachia* infected and uninfected RML 12 *Aedes albopictus* Mosquito cells.

We thank BEI Resources, American Type Culture Collection (ATCC), USA for kindly providing MRA 975 Human Hepatocyte cells.

References

1. Bruno Lemaitre and Jules Hoffmann. (2007). The Host Defense of *Drosophila melanogaster*. Annual Review of Immunology, 25: 697-743.
2. Conor. J. McMeniman, Amanda. M. Lane, Amy. W. C. Fong, Denis. A. Voronin, InakiIturbe-Ormaetxe, Ryuichi Yamada, Elizabeth. A. McGraw and Scott. L. O'Neill. (2008). Host Adaptation of a *Wolbachia* Strain after Long-Term Serial Passage in Mosquito Cell Lines. Applied and Environmental Microbiology, 74(22): 6963–6969.
3. Henk. R. Braig, Weiguo Zhou, Stephen. L. Dobson and Scott. L. O'Neill. (1998). Cloning and Characterization of a Gene Encoding the Major Surface Protein of the Bacterial Endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology*, 180(9): 2373–2378.
4. Hiroaki Noda, Takeharu Miyoshi and Yoko Koizumi. (2002). In vitro Cultivation of *Wolbachia* in Insect and Mammalian Cell Lines. In Vitro Cellular and Developmental Biology. Animal, 38(7): 423-427.
5. Jason. L. Rasgon, Courtney. E. Gamston and XiaoxiaRen. (2006). Survival of *Wolbachia pipientis* in Cell-Free Medium. Applied and Environmental Microbiology, 72(11): 6934–6937.
6. Lauren. M. Hedges, Jeremy. C. Brownlie, Scott. L. O'Neill and Karyn. N. Johnson. (2008). *Wolbachia* and Virus Protection in Insects. Science, 322(5902): 702.
7. Luis Teixeira, Alvaro Ferreira and Michael Ashburner. (2008). The Bacterial Symbiont *Wolbachia* Induces Resistance to RNA Viral Infections in *Drosophila melanogaster*. PLOS Biology, 6(12): 2753-2763.
8. S. L. O'Neill, M. M. Pettigrew, S. P. Sinkins, H. R. Braig, T. G. Andreadis, R. B.Tesh. (1997). In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Molecular Biology, 6(1): 33-39.
9. Sinkins. S. P. and Godfray. H. C. J. (2004). Use of *Wolbachia* to drive nuclear transgenes through insect populations. Proceedings of the Royal Society, 271: 1421–1426.
10. Stephen. L. Dobson, Eric. J. Marsland, Zoe Veneti, Kostas Bourtzis and Scott. L. O'Neill. (2002). Characterization of *Wolbachia* Host Cell Range via the In Vitro Establishment of Infections. Applied and Environmental Microbiology, 68(2): 656–660.
11. Weiguo Zhou, Francois Rousset and Scott O'Neill. (1998). Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proceedings of the Royal Society. B, 265: 509-515.

****

How to cite this article:

Kishore. V. P. *et al*.2016, Analysis of Human Hepatocyte Cell Lines For In Vitro Culture and Establishment of *Wolbachia*. *Int J Recent Sci Res.* 7(11), pp. 14177-14180.