J. ent. Res., 36 (2) : 119-122 (2012)

Long PCR : A sensitive PCR protocol for amplification of *Wolbachia* endosymbiont in Indian honey bees

N.M. Guruprasad, Sumithra, L. Mouton[**] and H.P. Puttaraju[*]

Department of Sericulture & Biological Sciences, Bangalore University, Bangalore - 560 056, Karnataka, India

ABSTRACT

A polymerase chain reaction (PCR) method was used to screen *Wolbachia* infection by using wsp (*Wolbachia* surface protein) primers. A nested polymerase chain reaction (Standard PCR) protocol yielded false negative results in the Indian honey bees *Apis millifera*, *Apis dorsata* and *Apis cerana*. This may be due to lower titer of *Wolbachia* or to factors that interfere with amplification of *Wolbachia* DNA. We developed a more sensitive method of amplification based on a Long PCR protocol using a proof reading enzyme.

Key Words : *Wolbachia*, Polymerase Chain Reaction (PCR), Standard PCR, Long PCR.

Endosymbionts of the genus *Wolbachia* (Rickettsiaceae) infect many arthropods and some nematode species. These maternally-transmitted symbionts induce various phenotypic effects on their hosts. In filarial nematodes, *Wolbachia* are mutualist since they are required for normal development and reproduction. In arthropods, *Wolbachia* infections are associated with a variety of reproductive anomalies in the host, including cytoplasmic incompatibility among populations of the same species (Guruprasad, 2009; Laven, 1951; Laven, 1967; Puttaraju and Prakash, 2005a; Puttaraju and Prakash, 2005b; Puttaraju and Prakash, 2005c) or between closely related species (Breeuwer and Werren, 1990; Breeuwer *et al.* 1992), thelytoky parthenogenesis in parasitoid wasps (Rousset *et al.* 1992; Sambrook *et al.* 1989), male killing in coccinellid beetles (Hurst *et al.* 1999), and feminization of genetic males in isopods (Rousset *et al.* 1992). In few cases, *Wolbachia* is obligatory for normal fecundity in the parasitoid *Asobara tabida* and in the bedbug *Cimex lectularis* (Dedeine *et al.* 2001; Hosokawa *et al.* 2010). *Wolbachia* are widespread symbionts. For example, 76% of all arthropod species tested by Jeyaprakash and Hoy (2000) and 19% of North American insect species (Werren *et al.* 1995a) were found to be infected.

Wolbachia are intracellular symbionts. They are found mainly in reproductive apparatus of their hosts. They cannot be cultured in defined media and detecting within infected gonadal cells may be time consuming. Therefore, detection of *Wolbachia* infection has been largely based on amplification of *Wolbachia* DNA using specific polymerase chain reactions. Primers designed from the *16S ribosomal DNA* (O'Neill *et al.* 1992) or from the nuclear genes *ftsZ* (Werren *et al.* 1995b) and *wsp* (*Wolbachia* surface protein) (Jeyprakash and Hoy, 2000; Zhou *et al.* 1998) have been used to amplify *Wolbachia* DNA from a diverse array of arthropods.

Preliminary work indicated that Standard PCR produced a high frequency of false positive results of *Wolbachia* infection in sericulture insects, so we investigated the efficiency and sensitivity of the Long PCR protocol. The Long PCR which uses two enzymes (*Taq* and *Pwo*) consistently amplified *Wolbachia* DNA and revealed that 49% of 35 arthropod species tested positive, which is considerably higher than the rate of 31% obtained previously obtained by using Standard PCR (Sumithra, 2009; Prakash and Puttaraju, 2007). The Long PCR protocol has been shown to more reliably amplify DNA than Standard PCR because two DNA polymerases are incorporated, one of which with a proof-reading activity (Barnes, 1994). Jeyaprakash and Hoy, (2000) found that Long PCR improves the likelihood of detecting *Wolbachia* DNA (intracellular symbionts of arthropods) when mixed with host insect DNA, a situation comparable to the situation

*Corresponding E-mail: puttarajuhp@hotmail.com
**Université de Lyon, Université Lyon1, Laboratoire de Biométrie et Biologie Evolutive, UMR CNRS, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

in which plant, psyllid or parasitoid DNA would be mixed with greening DNA. These authors found that the Long PCR protocol was approximately six orders of magnitude more sensitive than Standard PCR for amplifying *Wolbachia* DNA.

When Standard PCR protocol, was tried false negative results were frequently encountered in detecting *Wolbachia* in honey bees. Indeed, during this study we were unable to obtain *wsp* amplification in individuals of honey bees using the standard primers used to amplify the *wsp* gene (primers 81F and 691R; Zhou *et al.* 1998), prompting us to evaluate a different PCR based procedure called Long PCR. Long PCR consistently amplified 76% of sixty three arthropod species (Jeyprakash and Hoy, 2000).

MATERIALS AND METHODS

Honey bees collection and preservation : Honey bees were collected in Bangalore University campus and frozen at-80° until further use for DNA isolation and subsequent screening for the presence of *Wolbachia*.

DNA Isolation : The DNA from an individual insect was extracted following the standard phenol, chloroform, isoamyl alcohol (24:24:1) extraction and purification methods as described in Sambrook *et al.* (1989). The genomic DNA was quantified on 0.8% agarose gel.

PCR diagnosis

Standard PCR : A PCR assay based on the amplification of the published *Wolbachia* specific sequence primers was used (Zhou *et al.* 1998). The genomic DNA was resuspended in 50 µl of TE (10 mM Tris-HCl. 1 mM EDTA , P^H 8.0). The polymerase chain reaction (PCR) assay was carried out based on specific amplification of the *Wolbachia wsp* (*Wolbachia* surface protein) gene using the primers 81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC-3') and 691R (5'- AAA AAT TAA ACG CTA CTC CA-3') (Zhou *et al.* 1998). The PCR was carried out with the PTC 200 of MJ Research Thermocycler, in 25 µl reaction mixture containing 2.5 µl of 10 × PCR buffer, 0.5 µl of dNTP's (10 mM each), 2.5 µl of 2.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (New England Biolabs, England), 1 µl of both forward and reverse primer (5 pmol), 20 ng template DNA. The PCR was carried out with a cyclic condition of initial denaturation step at 94°C for 5 min followed by 35 cycles with denaturation step at 92°C for 1 min, annealing 55°C for 1.30 min extension 72°C for 1.15 min, final extension at 72°C for 10 min. The PCR products were checked by electrophoresis on 1.5% agarose gel running in 1 × TBE (89.2 mM Tris HCl, 88.9 mM Boric acid and 2 mM disodium EDTA) buffer for a length of about 5 cm with a constant voltage of 70 V. The gel was stained with 0.5 µg/ml ethidium bromide prior to casting. Gel documentation was done by using Alpha digidoc documentation system.

Long PCR : Long PCR was performed in a 50 ml volume containing 50 mM Tris (pH 9.2), 16 mM ammonium sulphate, 1.75 mM $MgCl_2$, 350 mM dATP, dGTP, dCTP, dTTP, 800 pM of primers (81F and 691R), 1 unit of Pwo (*Pyrococcus woesei*) and 5 units of Taq DNA polymerases (Barnes, 1994). The Long PCR was carried out using three linked profiles over 35 cycles; (1) 1cycle of denaturation at 94°C for 2 mins, (2) 10 cycles each consisting of denaturation at 94°C for 10 s, annealing at 59°C for 30 s and extension at 68°C for 1 min, and (3) 25 cycles each consisting of denaturation at 94°C for 10 s, annealing at 59°C for 30 s and extension at 68°C for 1 min, plus an additional 20 s for every consecutive cycle between 11 and 36 for both the primers. The amplified PCR products were separated on a 1.5% agarose 1 × TBE gel stained with 0.5 µg/ml of ethidium bromide. Documentation was done by using Alpha digidoc documentation system.

RESULTS AND DISCUSSION

Amplification of *Wolbachia* endosymbiont from honey bees *Apis millifera, Apis dorsata* and *Apis cerana* by using *wsp* primers using the standard PCR protocol produced false negative results. In a previous study, Wenseleers and Billen (2000) had the same difficulty to amplify *Wolbachia* in a population of *Apis millifera* by using Standard PCR based on the *16S rDNA* gene.

An attempt has been made to amplify *Wolbachia* DNA from the honey bees by using Long PCR. Long PCR successfully obtained from the three honey bee populations *Apis millifera, Apis dorsata* and *Apis cerana* consistently gave positive results producing a band size of 600bp with high band intensity (Fig. 1). Long PCR protocol produced bright bands indicating that the Long PCR protocol reduced the frequency of false negatives. A sensitivity analysis conducted earlier by Jeyaprakash and Hoy (2000) based on the amplification of the *wsp* and the *16S rDN* genes in diverse arthropod species indicated that the Long

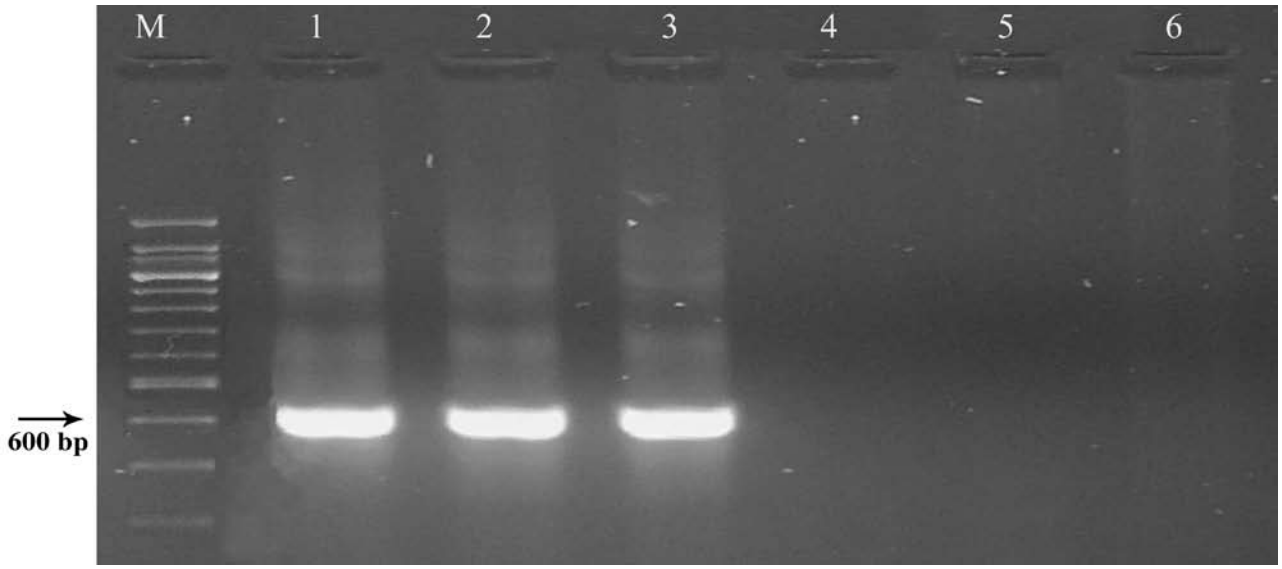

Fig. 1. *Wolbachia* wsp gene amplification in honey bees. Lane M-Molecular weight marker, lanes 1-3, Long PCR amplification of *A. millifera*, *A. dorsata* and *A. cerana* and lanes 4-6, Standard PCR amplification of *A. millifera*, *A. dorsata* and *A. cerana*

PCR is approximately six to eight times more sensitive than standard PCR. The failure of Standard PCR to amplify *Wolbachia* DNA could be due to inhibition of the reaction or to the lower titer of *Wolbachia* DNA resulting in non optimal primer DNA template ratio. Prolonged denaturation could cause breakage of the DNA template and depurination of bases, which could stop the extension by Taq (Chu *et al.* 1986; Hoy *et al.* 2001).

The Long PCR procedure utilizes both Taq and a thermostable DNA proof reading polymerase (Pfu, Vent or Deep Vent), which exhibits a 3- to 5-exonuclease activity (Barnes, 1994). The Pwo DNA polymerase from *Pyrococcus woesei* shares 100% DNA sequence identity with Pfu from *P. furiosus* and exhibits similar exonuclease activity. The successful amplification of *Wolbachia* DNA mixed with arthropod genomic DNA by the Long PCR may be due to the exonuclease activity of the Pwo polymerase (Jeyprakasha and Hoy, 2000).

The Standard PCR protocol revealed a low sensitivity leading to high levels of false negative results. Long PCR, which incorporates a second DNA polymerase with proof reading activity yielded consistent results with orders of magnitude more sensitive than the Standard PCR. The sensitivity of Long PCR indicated that this procedure could be used to amplify other microbial DNA, in which Standard PCR gives false negative results. Our study indicates that Long PCR protocol with a proof reading enzyme along with the Taq DNA polymerase is more reliable and sensitive than the standard PCR.

REFERENCES

Barnes, W. 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from l bacteriophage templates. *Proc. Natl. Acad. Sci.*, USA, **91**: 2216-20.

Breeuwer, J.A.J. and Werren, J.H. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, **346**: 558-60.

Breeuwer, J.A.J., Stouthamer, R., Burns, D.A, Pelletier, D.A., Weisburg, W.G. and Werren, J.H. 1992. Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus Nasonia (Hymenoptera : Pteromalidae) based on 16 Sribosomal DNA sequences. *Insect Mol. Biol.*, **1**: 25-36

Chu, G., Vollrath, D. and Davis, R.W. 1986. Separation of large DNA-molecules by contour-clamped homogeneous electric-fields. *Science*, **234**: 1582-85.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E. and Bouletreau, M. 2001. Removing symbiotic Wolbachia bacteria specifically inhibits

oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci.*, *USA* **98**: 6247-52.

Guruprasad, N.M. 2009. *Wolbachia infection and its implication on population structure and dynamics of uzifly Exorista sorbillans* (*Diptera* : *Tachinidae*), Ph.D. Dissertation, University of Bangalore.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.Y. and Fukatsu, T. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *P. N. A. S.*, **107**: 769-74.

Hoy, M.A., Jeyaprakash, A. and Nguyen, R. 2001. Long PCR is a sensitive method for detecting *Liberobacter asiaticum* in parasitoids undergoing risk assessment in quarantine. *Biological Control*, **22**: 278-87.

Hurst, G.D.D., Jiggins, F.M., Schulenburg, J.H.G.VD., Bertand, D., West, S.A., Goriacheva, I.I., Zakharov, I.A., Werren, J.H., Stouthamer, R. and Majerus, M.E.M. 1999. Male-killing Wolbachia in two species of insect. *Proc. R. Soc. Lond. B.*, **266**: 735-40.

Jeyaprakash, A. and Hoy, M.A. 2000. Long PCR improves *Wolbachia* DNA amplification: WSP sequence found in 76% of sixty-three arthropod species. *Insect Mol. Biol.*, **9**: 393-405.

Laven, H. 1951. Crossing experiments with *Culex* strains. *Evolution*, **5**: 370-75.

Laven, H. 1967. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature*, **261**: 383-84.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. and Robertson, H.M. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. *Natl. Acad. Sci.*, *USA* **89**: 2699-702.

Prakash, B.M. and Puttaraju, H.P. 2007. Frequency of infection with A and B supergroup Wolbachia in insects and pests associated with mulberry and silkworm. *J. Biosci.*, **32**: 671-76.

Puttaraju, H.P. and Prakash, B.M. 2005a. *Wolbachia* and reproductive conflicts in the uzifly, Exorista sorbillans (Diptera : Tachinidae). Archieves of *Insect Biochemistry and physiology*, **60**: 230-35

Puttaraju, H.P. and Prakash, B.M. 2005b. Effects of *Wolbachia* in the silkworm parasitoid Exorista sorbillans (Diptera : Tachinidae). *J. Insect Sci.*, *USA* **30**: 1-7.

Puttaraju, H.P. and Prakash, B.M. 2005c. Effects of *Wolbachia*-Targeted tetracycline on Host-parasitoid-symbiont interaction. *European J. Ent.*, **102**: 669-74.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. and Solignac, M. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. London*, *Ser. B*, **250**: 91-98.

Sambrook, J., Fritsch, E.F. and Miniatis, T. 1989. *Molecular cloning* : *A Laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stouthamer, R., Breeuwer, J.A.J., Luck, R.F. and Werren, J.H. 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature*, **361**: 66-8.

Stouthamer, R., Luck, R.F. and Hamilton, W.D. 1990. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Prc. Natl. Acad. Sci. USA*, **87**: 2424-27.

Sumithra. 2009. *Molecular phylogenetic analysis of Wolbachia endosymbiont in the insect pests of Sericulture*, Ph.D. Dissertation, University of Bangalore.

Wenseleers, T. and Billen, J. 2000. No evidence for Wolbachia-induced parthenogenesis in the social Hymenoptera. *J. Evol. Biol.*, **13**: 277-80.

Werren, J.H., Windsor, D. and Guo, L.R. 1995a. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B.*, **262**: 197-204.

Werren, J.H., Zhang, W. and Guo, L.R. 1995b. Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. Proc. *R. Soc. London*, *Ser. B.*, **261**: 55-63.

Zhou, W., Rousset, F. and O'Neill, S.L. 1998. Phylogeny and PCR-based classification of *Wolbachia* strain using wsp gene sequences. *Proc. R. Soc. London*, *Ser. B*, **265**: 509-15.

(Accepted : April 15, 2012)