

# **Workshop on “PCR-based Detection of Silkworm Diseases”**

September 18 and 19<sup>th</sup>, 2014

## **Handout-1**



**Seri-biotech Research Laboratory (SBRL),  
Central Silk Board, Kodathi, Bangalore**

**Day 1: September 18, 2014**

## **Development of a multiplex polymerase chain reaction (PCR) technique for the simultaneous detection of microsporidians, nucleopolyhedrovirus, and densovirus infecting silkworms**

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### **Introduction**

India is the homeland of all the four varieties of natural silks: Mulberry, Tasar, Eri and Muga. Mulberry silk produced by the silkworm, *Bombyx mori* constitutes 90% of the total silk production. Sericulture is an agro-based industry where six million people are engaged in various sericulture activities in India. It is a prerequisite to ensure the silkworms free of pathogens before being supplied to farmers. Lack of accurate disease detection techniques in sericulture causes severe spread of diseases leading to crop loss of 30-40%. The silkworm, *B. mori* and other related species are susceptible to different kinds of diseases caused by pathogens. The most common pathogens infecting them are microsporidians including *Nosema bombycis*, nucleopolyhedrovirus (NPV) and densovirus, infectious flacherie virus (IFV), cytoplasmic polyhedrovirus (CPV), and bacteria. In India, conventional microscopy is widely used for the detection of silkworm pathogens. Though microscopy is inexpensive and easy to use, it has inherent problems such as late detection (polyhedra and microsporidian spores are visible only after 3–8 days of onset of infection); and in the case of DNV infection an electron microscope is required. Hence, there is need to develop a specific and accurate early detection method, which prompted us to undertake the present work. This should enable the detection of major pathogens of silkworms and help researchers and farmers for effective disease management in sericulture.

### **Technology**

A multiplex polymerase reaction (PCR) - based method was developed for the simultaneous detection of three main pathogens infecting the silkworm, *Bombyx mori* (Nuclearpolyhedrosis virus, (NPV), Densonucleosis virus (DNV) and a fungus *Nosema bombycis*). Each of the three primers exclusively amplified the target gene of specific pathogen. The specificity of this technique for the

detection of three pathogens was found to be good. The lower limit of detection of this test was estimated to be 1500 spores and 3700 polyhedra per 20 mg of tissue for microsporidian and NPV respectively, whereas DNV was detected with relatively very low quantity of DNA. The multiplex PCR assay is an effective tool for the early detection of three main pathogens from naturally infected silkworms. The system also can accommodate more number of pathogens and can be used to detect same or similar pathogens infecting other silkworms/insects.

### **Salient Features**

1. PCR -based detection
2. Simultaneous detection in a single tube
3. Early detection of pathogens
4. Extended to any silkworms and insects infected by same pathogens
5. Complement the activities of CSB for its regular disease detection program and quarantine activities
6. Patent pending with National Research Development Organization (NRDC), Govt of India.
7. Published in a reputed international journal

G. Ravikumar , S. Raje Urs, N.B. Vijaya Prakash, C.G.P. Rao, and K.V. Vardhana. *Journal of Invertebrate Pathology*. 107 (2011) 193–197.

## **PROTOCOL**

### **PCR components:**

**DNA sample:** This is the template to which the primers will anneal and DNA polymerase will create copies of the target sequence.

**PCR buffer:** Creates a stable environment in which PCR can most effectively occur.

**MgCl<sub>2</sub>:** Acts as a catalyst for DNA polymerase.

**dNTPs:** Provides bases for use by DNA polymerase to create DNA.

**Primers:** Used to anneal to DNA to create copies of the target sequence.

**DNA polymerase:** Creates copies of target DNA.

**Nuclease-free water:** Brings the reaction up to the appropriate volume.

**Thermocycler:** This is the machine used to raise and lower the temperature of the samples throughout the PCR cycles.

**Other Components:**

0.2 ml PCR tubes

Pipettes

Pipette tips

Microcentrifuge

**Primers:****1. *Microsporidian SSU-rRNA:***

Forward: 5'-ACCAGGTTGATTCTGCCTGA-3',

Reverse: 5'-GTTGAGTCAAATTAAGCCG-3'

**2. *NPV polyhedrin:***

Forward: 5'-GTIAARCCIGAYACIATGAA-3',

Reverse: 5'-GCRAAYTCYTTIATYTTTRAAIAC-3'

(I: deoxyinosine; R: A, G; Y: T, C)

**3. *DNV 2 & 3:***

Forward: 5'-AGAACCGCAAGTAATCAAGC-3'

Reverse: 5'-CATTATATCTCCATTAGTTCC-3'

**DNA Extraction**

DNA was isolated from microsporidian/NPV/DNV infected silkworms using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocols.

**Singleplex PCR for Microsporidians, NPV, and DNV**

10 x Taq Buffer: 1  $\mu$ l

2.5 mM dNTPS: 1  $\mu$ l

25 mM MgCl<sub>2</sub>: 1  $\mu$ l

10  $\mu$ M Primer-F-Micro. or NPV or DNV: 0.5  $\mu$ l

10  $\mu$ M Primer-R-Micro. or NPV or DNV: 0.5  $\mu$ l

DNA extracted from silkworm infected with Micro.: 125 or NPV: 150 or DNV: 50 ng

Taq DNA Polymerase: 0.5 U

dH<sub>2</sub>O: q.s.

Total: 10  $\mu$ l

## Multiplex PCR for Microsporidians, NPV, and DNV

10 x Taq Buffer: 3.5  $\mu$ l  
 2.5 mM dNTPS: 2  $\mu$ l  
 25 mM MgCl<sub>2</sub>: 2.5  $\mu$ l  
 10  $\mu$ M Primer-F: 2  $\mu$ l (NPV)  
 10  $\mu$ M Primer-R: 2  $\mu$ l (NPV)  
 10  $\mu$ M Primer-F: 1  $\mu$ l (Micro.)  
 10  $\mu$ M Primer-R: 1  $\mu$ l (Micro.)  
 10  $\mu$ M Primer-F: 0.5  $\mu$ l (DNV)  
 10  $\mu$ M Primer-R: 0.5  $\mu$ l (DNV)  
 DNA extracted from silkworm infected with Micro.: 170, NPV: 220 and DNV: 50 ng  
 Taq DNA Polymerase: 2.5 U  
 dH<sub>2</sub>O: q.s.  
 Total: 28.5  $\mu$ l

(A negative control also will be included)

### PCR Reactions:

1. **Preincubation:** DNA is dissociated into single linear strands, primers are dissociated and DNA polymerases are activated.
2. **Denaturation:** DNA and primers are dissociated.
3. **Annealing:** Primers adhere to DNA. This step results in the creation of primer-DNA hybrids.
4. **Extension:** DNA polymerase creates new DNA at the primer sites in a 5'  $\rightarrow$  3' direction. Denaturation, annealing and extension steps are repeated to amplify the DNA fragments.
5. **Final extension:** The final extension of any remaining single DNA strands is made.
6. **Hold:** After PCR, the product is kept at 4°C for better preserving the newly-made DNA.

### PCR Conditions:

**Preincubation at 94°C:** 2 min

**Denaturation at 94°C:** 40 sec

**Annealing at 48°C:** 45 sec      x 30 cycles

**Extension at 72°C:** 40 sec

**Final extension at 72°C:** 5 min

**Hold at 4°C:** Infinity

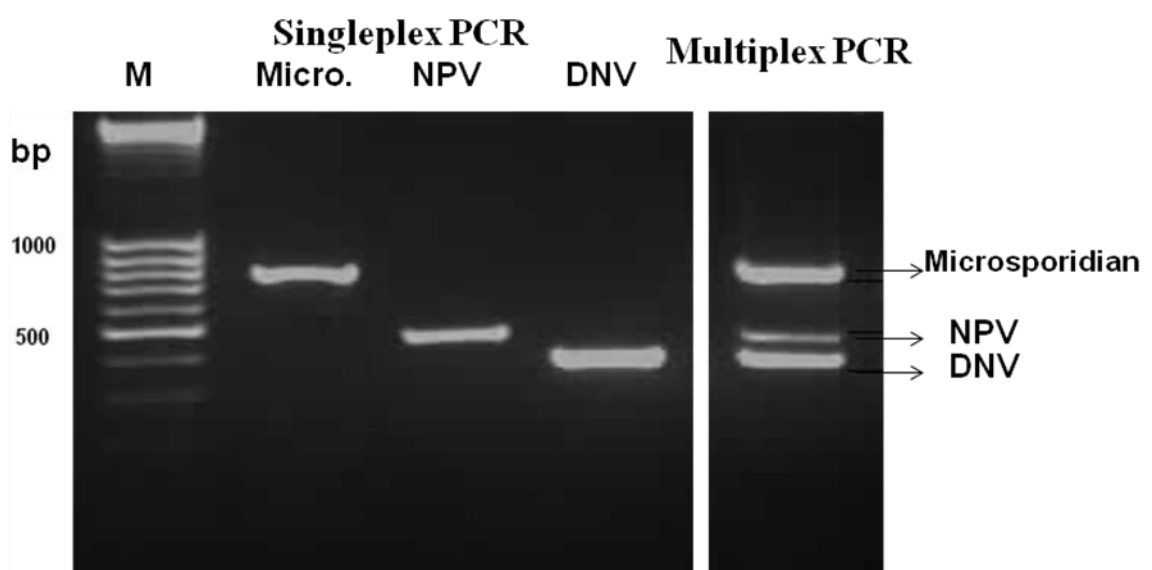
### Results by agarose gel electrophoresis:

1. Cast an agarose gel
2. Load samples and DNA Marker onto the gel
3. Perform electrophoresis
4. Read the gel under UV light to detect DNA bands
6. Record results

(Please see Annexure 1 for details)

### Results

Single and multiplex PCR, using three primer pairs, two of which were designed from the conserved regions of 16S small subunit ribosomal RNA gene of microsporidians, and polyhedrin gene of NPVs respectively, and a third primer pair designed from the internal sequences of *B. mori* DNVs (*BmDNV*), showed discrete and pathogen specific PCR products. The assay showed high specificity and sensitivity for the pathogenic DNA. Under optimized PCR conditions, the assay yielded a 794 bp DNA fragment from *Nosema bombycis*, 471 bp fragment from *B. mori* NPV (*BmNPV*) and 391 bp fragment from *BmDNV*. Further, this detection method can be applied to other silkworm species in detecting same or similar pathogens infecting them. This method is a valuable supplement to the conventional microscopic diagnostic methods and can be used for the early detection of pathogens infecting silkworms. Furthermore it can assist research and extension centers for the safe supply of disease-free silkworms to farmers.



**Single and multiplex PCR of silkworm pathogens. M: Size Marker**

## ANNEXURE 1

### Components used for PCR results:

1. Agarose: This is galactose polymer which is cast as a gel. The gel is a matrix through which DNA passes. When an electrical current is passed through the gel, DNA moves in the agarose from the negative pole to the positive pole and is separated according to size; the longer DNA fragments take longer to migrate than the shorter fragments.
2. Electrophoresis machine: This device creates an electrical current. DNA, being negatively charged, migrates from negative to positive.
3. TAE buffer: This buffer is used in the electrophoresis machine to conduct current through the gel. It is also used to prepare the agarose gel.
4. DNA ladder: This provides standards for different-sized DNA fragments, which can be compared to sample bands to obtain an approximate size for sample DNA fragments.
5. Loading dye: This is added to the DNA samples and DNA ladder to allow visualization of sample when loading and migration of the sample during electrophoresis. A common dye is bromophenol blue, which runs at the same speed as a 300 bp DNA fragment.
6. Ethidium bromide: Binds to the major groove of DNA and fluoresces under ultraviolet light.

### Materials and equipments:

Graduated cylinder  
Glass flask  
Agarose  
Gel plate with well comb  
Concentrated TAE buffer  
dH<sub>2</sub>O  
Pipettes and pipette tips  
Ethidium bromide  
PCR product  
DNA loading dye  
DNA ladder  
Electrophoresis chamber  
Ultraviolet light source  
Microwave

### Preparing an agarose gel:

1. Prepare a gel plate with comb
2. Measure out 100 ml of 1× TAE buffer in the glass flask
  - a. Dilute concentrated TAE in dH<sub>2</sub>O
3. Measure out and add 1 g of agarose to the glass flask

4. Microwave the flask for 1 minute
  - a. Watch to make sure the liquid does not boil over
5. Swirl the mixture and microwave again for 1 minute
  - a. Repeat if agarose is not fully dissolved
6. When the agarose is fully dissolved (no pieces of agarose visible in the liquid), then run the flask under cold water to cool the mixture
  - a. Do not get water in the flask
  - b. Swirl the flask while cooling
7. When the mixture is warm to the touch (not hot), add 2  $\mu$ l of ethidium bromide solution
8. Swirl the flask and pour the liquid into a gel plate
9. Allow agarose to harden

### **Performing gel electrophoresis:**

1. Place the hardened agarose gel into the electrophoresis machine
  - a. Make sure the wells are facing toward the negative side
2. Fill the electrophoresis machine with 1 $\times$  TAE buffer to the level indicated
3. Carefully remove the comb from the agarose gel
  - a. Lift straight up to avoid tearing the wells
4. Add 2  $\mu$ l DNA loading dye to your PCR sample:
  - a. Mix the PCR sample by flicking with your finger
  - b. Pipette the sample several times to mix with the DNA loading dye
5. Add the 6  $\mu$ l of PCR sample mixed with loading dye to a well:
  - a. Brace the pipette against your non-dominant hand to steady pipetting and prevent going through the well
  - b. Take your time. Pipette slowly into the well to avoid the sample shooting out of the well
  - c. Perform this for all samples, including negative and positive controls
  - d. It is recommended to add the negative control first and the positive control last
  - e. Make a note of the locations of your samples in the wells
6. Add 6  $\mu$ l of DNA ladder mixed with loading dye to the first well
7. Close the lid on the electrophoresis machine, and attach the electrodes to the power source
8. Perform electrophoresis at 100 V under constant voltage:
  - a. The run should take about 45 minutes
  - b. Watch to make sure the dye does not run off the gel
9. After running, view the gel under the ultraviolet light
10. Check the position of bands against each other and against the DNA ladder

(Courtesy: IVSProgram, Summer Research 2013)

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