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Molecular Characterization of Pathogenic Bacteria Isolated from Flacherie Disease of Silkworm, *Bombyx mori*

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Abstract

Flacherie disease is characterized by a condition which is generally called digestive disorder or septicemia in silkworm. The presence of different groups of bacteria are considered as contributing factors for the bacterial flacherie. During infection different species viz., *Streptococcus sp. Micrococus sp. Bacillus sp*. and *Serratia marcescens* were found in the flacherie affected silkworm. Their occurance may be due to involvement of infectious flacherie virus, densonucleosis and cytoplasmic polyhedrosis and coccus spp. of bacteria or mixed infection by both bacteria and virus. As per the sequencing of organisms, the isolated bacteria from the infected silkworm *viz.,* isolated bacteria 1 had 95 per cent similarity with *Lysinibacillus sphaericus,* isolated bacteria 2 had 99 per cent similarity with *Alcaligence faecalis* strain SDK 2 which was found for the first time in the infected silkworm. Isolated bacteria 3 had 99 per cent silmilarity with *Bacillus subtilis* C11. The presence of these isolates are found to be attributing pathogenic factors in addition to rearing factors in Eastern dry zone of Karnataka.

Silkworm, *Bombyx mori* L. is an important economic lepidopteran insect and utilized for the commercial production of natural silk fiber *viz.,* Queen of Textile. Its application in all over the world finely contributed as a powerful laboratory model for the basic and applied research in biology. India occupies second position in raw silk production in the world, through its productivity per unit area is low compared to sericulturally advanced countries. Country's raw silk production has reached a new height of 28708 MT with a growth rate of 8.4 per cent over the previous year (Anonymous, 2014a).

Silkworm being poikilothermic, responds very quickly to environmental changes particularly to temperature, relative humidity, quality of leaf and density of silkworm which often leads to the occurrence of infectious diseases caused by virus, bacteria, fungi and protozoa. Flacherie disease is characterized by a condition which is generally called digestive disorder or septicemia in silkworm. The attributing factors for the bacterial flacherie infection are *Streptococcus sp., Micrococus sp., Bacillus sp*. and *Serratia marcescens,* whereas flacherie caused by viruses involves infectious flacherie virus, densonucleosis and cytoplasmic polyhedrosis and coccus spp. of bacteria or mixed infection by both bacteria and virus (Selvakumar and Datta, 2013).

In India, the prevalence of flacherie is 15-40 per cent crops (Anonymous, 1994b). Among flacherie, microbial pathogen, the bacteria prevails in 57.22 per cent crops (Samson *et al.,* 1990) while viruses such as *Bm*IFV and *Bm*DNV reported from 43.05 per cent crops (Anonymous, 1994c). Therefore there is a need to identify the association of other micro organisms involved in the falcherie disease through tools of molecular biology and bioinformatics which allow the identification of new groups of microbes which have been unexplored till today. Probes using target sequences of 16SrRNA gene have been widely used as a molecular clock to estimate relationship among the bacteria and identification of unknown bacteria upto genus and species level (Sacchi *et al.,* 2002).

The research work was carried out at the, Department of Sericulture and Department of Agricultural Microbiology, College of Agriculture, University of Agricultural Sciences, GKVK, Bengaluru-65. The procedure and techniques adopted during molecular characterization of pathogenic bacteria isolated from flacherie disease of silkworm, *Bombyx mori* are presented below.

The silkworms exhibiting the symptoms of flacherie disease were collected from farmers rearing house and the pathogens were isolated from diseased

silkworm with standard procedure (Govindan *et al*., 1998). Further, sub-culturing of isolated pathogens was done by streak plate method and incubated at temperature of 37 ÚC for 2-3 days depending upon the colony growth.

Total genomic DNA of the three bacterial isolates was extracted by alkaline lysis method. The bacterial isolates were grown in Luria broth for 48 hours at 30 ÚC and 3 to 5 ml bacterial culture were pelleted by centrifugation at 10,000 rpm. The bacterial pellet was re-suspended in 250µl of extraction buffer (10mM Tris HCl, pH 8.0, 20mM EDTA and 250mM NaCl) and incubated at 60 ÚC for 30 minutes for lyses.

Later, the extract was added 100µl of 5M potassium acetate solution and placed on ice for 10 minutes for precipitation of protein and carbohydrates and the clear supernatant was collected by centrifugation. The DNA was precipitated by adding equal volume of ice cold isopropanol, and DNA pellet was collected by centrifugation at 12,000 rpm. The pellet was washed twice with 70% ethanol, air dried, and dissolved in 10mM TE (10:1) buffer stored in aliquots at -20 ÚC. The quality and quantity of the isolated DNA was checked using UV spectrophotometer at 260/280 nm wave length.

A 26 bp of forward primer 5' GTTAGATCTTGGCTCAGGACGAACGC 3' and 24 bp of reverse primer 5' GATCCAGCCGCACCTTCCGATACG 3' already reported for 16S rRNA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov) were custom synthesized by Sigma-Aldrich (Sigma, USA) and diluted accordingly for the PCR reactions. Annealing temperature for primer pair were standardized and PCR was performed in a 40µl reaction volume containing 1X buffer with $MgCl_2$ (1.5mM), dNTPs (200µM), forward and reverse primers (0.5µM each), *Taq* DNA polymerase (1U Genei Bangalaore) and template DNA (50ng). Amplification was carried out with an initial denaturation at 96 ÚC for 3 minutes followed by 35 amplification cycles consisting of 94 ÚC for 1 minute, 50 ÚC for 30 seconds and 72 ÚC for 1 minute and a final extension step at 72 ÚC for 10 minutes. Controls for PCR reactions were carried out with the same primers without providing template DNA. PCR products were separated on 1.0 per cent agarose gel and documented using gel documentation system Hero Lab, Germany.

Agarose gel electrophoresis was performed to resolve the amplified product using 0.8 per cent agarose in 1 X TAE buffer, 0.5 µg / ml of ethidium bromide, and loading buffer (0.25% bromophenol blue in 40% sucrose). Five µl of loading dye was added to 40 µl of PCR product and loaded to the agarose gel. Electrophoresis was carried out at 100V for 2 hours. The gel was visualized under UV light and documented using Alpha innotech Gel documentation unit. The visualized band was excised and purified by using gel extraction kit. The Gene JET™ Gel Extraction Kit (Thermo Scientific) was used for rapid and efficient puriûcation of DNA fragments from agarose gels. This is used for the elution of amplified product bacterial DNA.

Sequence results were analysed with online software of National Centre for Biotechnology Information (NCBI), USA. The BLAST (Basic Local Alignment Search Tool) search gave the partial length sequence homology with NCBI data (http//www.Ncbi.nlm.nih.gov/BLAST/).

As per the sequencing of organisms the isolated bacteria from the infected silkworm B1 (Isolated bacteria 1) had 95 per cent similarity with *Lysinibacillus sphaericus* C3-41 with Accession number CP00081.17 and basepair of 1041. B2 (Isolated Bacteria 2) had 99 per cent similarity with *Alcaligence faecalis* strain SDK 2 with accession number KM502543.1 and basepair of 784 and B3(Isolated Bacteria 3) had 99 per cent silmilarity with *Bacillus subtilis* C11 strain with accession number KU557409.1 and basepair of 656 (Plate 1 and 2).

These observations are in conformity with the finding of Mathavan *et al.* (1991) who reported that *Bacillus sphaericus* causes severe damage to the midgut cells of silkworm, the extent of decrease in the RNA and protein content of the columnar cells are remarkable.

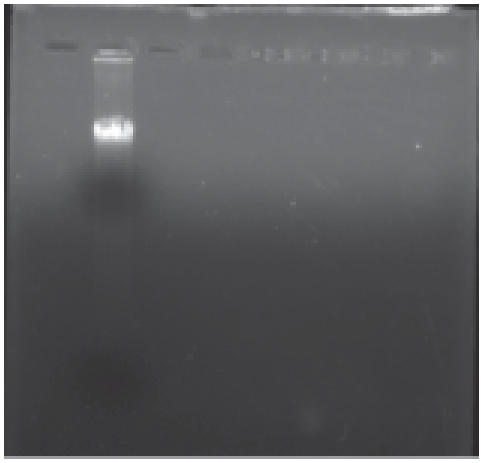

Plate 1. Genomic DNA of Bacteria 3 (*Bacillus subtilis)*

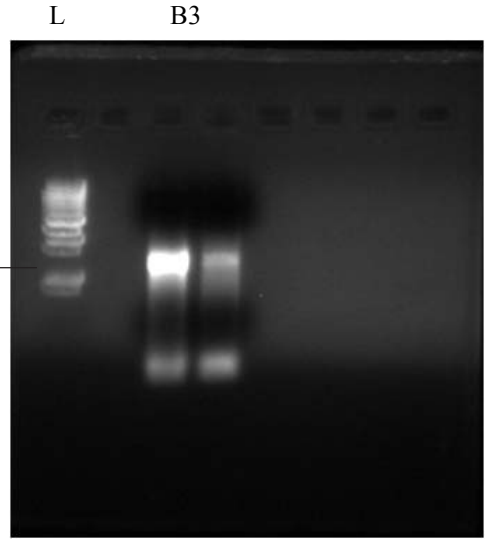

Plate 2: PCR amplification of 16SrRNA gene of Bacteria 3 (*Bacillus subtilis)*

Sakthivel *et al*. (2012) has reported bacteria as etiological agents of flacherie in silkworm and the organisms identified were *Bacllus Subtilis, Streptococcus pneumoniae, Staphylococcus aureus, E. coli, Pseudomonas fluorescence, Bacillus cereus* and *Klebsiella cloacae.*

Further, the most commonly associated bacteria with bacterial flacherie in India are species of *Streptococci, Staphylococci, Bacillus and Serratia* reported by Anitha *et al.* (1994). Manoj *et al*. (2013) isolated a bacterium from the eggs of the silkworm, and a gene encoding 16S rRNA was amplified using PCR, sequenced and the bacterium was identified as *Bacillus subtilis*.

In addition to *Bacillus subtilis and Bacillus sphaericus/Lysinibacillus sphaericus* a new pathogenic bacterium *viz.*, *Alcaligence faecalis* strain SDK 2 was isolated from the Flacherie /Mixed infection of Silkworm. It is confirmed from the microscopic observation that this organism is located in the esophagus and intestine of the nematodes *Steinernema feltiae, S. carpocapsae*, and *H. bacteriophora*. By Using *G. mellonella* larvae as a model, when the larvae were injected with 24,000 CFU of *Alcaligence faecalis* in their hemocoel, more than 96 per cent mortality was achieved after 24 h. Additionally, toxicity assays determined that 1 5Øßg of supernatant extract from *A. faecalis* MOR02 killed more than 70 per cent *G. mellonella* larvae 96 h after injection (Rosa Estela *et al.,* 2015).

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