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Identification and characterization of bacteria causing flacherie in mulberry silkworm, *Bombyx mori* L.

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ABSTRACT

Flacherie in silkworms is defined as a state of larval health typified by flaccid condition of the affected larvae and caused by non-occluded viruses and bacteria individually and in combination. Among the silkworm diseases, flacherie is one of the major contributing factors to cocoon crop loss in India. The present work aims to isolate pathogens responsible for inflicting bacterial infection in Bombyx mori and characterize the associated bacteria. Mid gut contents were dissected from the larvae exhibiting typical symptoms of flacherie. The same were subjected to serial dilution and plated onto nutrient agar plates. Five bacterial cultures were isolated in axenic form and identity of the same was affirmed by sequencing the 16S rRNA gene. EZ Taxon BLAST analysis revealed that they were exhibiting highest sequence similarity to Staphylococcus argenteus, Paracoccus niistensis, Bacillus carboniphilus, Enterococcus cecorum and Enterobacter asburiae. Staphylococcus argenteus and Bacillus carboniphilus were found to be pathogenic to silkworm and inflicted mortality up to a tune of 50%.

***Keywords*:** Bacteria, flacherie, koch postulates, molecular phylogeny and 16S rRNA gene sequencing

Sericulture is a potential source of income for the farming fraternity across many countries of the globe. Like many other insects, mulberry silkworm *B. mori* is also susceptible to a diverse range of micro organisms causing crop losses up to 20% (Jiang *et al*., 2013). Grasserie (viral disease), Muscardine (fungal disease), Pebrine (microsporidian infection) and Flacherie, a syndrome inflicted by non-occluded viruses, bacteria and both in combination are the frequently encountered diseases in silkworm. It is imperative to appropriately manage and prevent silkworm diseases for a successful harvest of a cocoon crop.

Among silkworm diseases, flacherie is one of the major diseases responsible for crop losses up to a tune of 10-15% (Bebitha *et al*., 2016). Symptoms of flacherie comprise loss in appetite, lethargy, hindered growth, thorax inflammation, emergence of brown specks on skin, rectal protrusion, chain type excreta, rupture of skin, discharge of brown fluid with foul odour and larvae turns black upon death (Zhang *et al*., 2013).

Members of *Aeromonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Staphylococcus* and *Streptococcus* have been reported to inflict bacterial flacherie and non occluded viruses (*Bm*IFV/*Bm*DNV) are accountable for viral flacherie in silkworm (Tao *et al*., 2011). The etiology of bacterial infections in silkworm is not completely understood as diverse bacterial strains are reported to be involved in causing the infection (Choudhury *et al*., 2002, Kaito *et al*., 2002). Isolation, identification and characterization of the etiologic agents responsible for the diseases are imperative in designing disease control strategies. The present study aims to isolate and identify pathogens responsible for causing bacteriosis in silkworm and characterize the associated bacteria.

MATERIALS AND METHODS

Isolation and culturing of bacteria

Diseased specimens (silkworms exhibiting typical symptoms of flacherie) were collected from farmers rearing houses of Murshidabad district during April, 2019. The same were surface sterilized with 0.5% sodium hypochlorite solution to remove the external micro flora. Subsequently, they were subjected to thorough rinsing with sterilized nuclease free water to remove traces of the sterilant.

The mid gut contents were extracted by dissection and the same were serially diluted (10^{-1} - 10^{-8}) in sterilized saline solution and 0.2 ml from each dilution was plated onto respective nutrient agar [Himedia (Cat No: M001)] plates. The inoculated plates were incubated at 28+2 °C for two days. The bacterial isolates were subjected to purification by repetitive streaking. Purity of the cultures was affirmed by examining the characteristics of the isolates *viz*., color, morphological aspects of the colony and by microscopic assessment. Pure cultures were maintained by refrigerating the same and periodical sub-culturing was performed every week.

Colony morphological characters were recorded by visual observation (Cappuccino and Sherman, 1988) whereas cellular morphological properties of the isolated strains were recorded upon Gram staining followed by visualization under a phase contrast microscope. Gram-staining of the bacterial isolates under test was performed using a Gram-staining kit [HiMedia (Cat No: K001)] following manufacturer's instructions.

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Molecular identification of bacteria

Identities of the isolated bacteria were determined by sequencing the 16S rRNA gene. Well grown bacterial colony was picked with a sterilized inoculation loop and dispersed in 50 micro liters of nuclease free water. The bacterial suspension was lysed in a thermocycler (Eppendorf) at 92 °C for 10 minutes and the same was made use of as a template. The primers employed were F-27 and R-1489 [Brosius *et al*., 1978; Lane *et al*., 1985; Imhoff *et al*., 1998; Imhoff and Pfennig, 2001]. The PCR reaction mixture was prepared by adding 1 ìl each of forward and reverse primers, 2.5 ìl template DNA, 8 ìl DNase free water and 12.5 ìl master mix (Himedia). The mixture was subjected to PCR conditions as delineated by Anil Pappachan *et al*., 2019. Upon completion of the reaction, the amplicons were refrigerated until further use.

Agarose gel electrophoresis

Five micro liters each of the PCR products and DNA ladder (Himedia 100bp DNA Ladder, MBT130) were electrophoresed on a 2% agarose gel, EtBr stained and the same was observed in a gel documentation system (EZ imager of Biorad).

16S rRNA gene sequencing

Subsequent to confirmation of amplification, the amplicons were subjected to sequencing at M/s. Xcelris Labs Limited, Ahmedabad, Gujarat.

BLAST search

Nearest phylogenetic relatives were determined by submitting the sequence to EzTaxon platform. EzTaxon platform is a versatile tool which aids in estimating the similarity of submitted test 16S rRNA sequence with sequences of type strains (Yoon *et al*., 2017).

Bioassay studies

A bioassay was conducted during July-August, 2019 to ascertain the infectivity of the bacteria isolated. All the bacterial strains that were isolated were inoculated at a concentration of 1 x 10^7 cfu/ml to *B. mori* (M.Con.4), healthy silkworms on the first day of the III instar by leaf disc method. Enumeration of bacteria was determined by serial dilution technique. Three replications of fifty larvae were maintained for each treatment. 200 µl of inoculums was smeared uniformly on a mulberry leaf disc, measuring 7.5 cm in diameter. Each batch of 50 larvae was fed with 2 such leaf discs. A control batch was also maintained devoid of inoculation but fed with leaves smeared with distilled water. Mortality due to flacherie was recorded based on visual symptoms and microscopic examination. To confirm the pathogenicity of the bacterial isolates, infectivity test was performed employing Koch's postulates. Post inoculation, flacherie infected larva from the treatment batches were collected and bacteria were re-isolated and purified following the same methodology as delineated above.

RESULTS AND DISCUSSION

Five bacterial pure cultures were isolated from gut contents of flacherie infected silkworm larvae and strain numbers (Bac 1-5) were designated. Different aspects of colony morphology such as colour, pigmentation, texture, margin, size, elevation and shape of all the isolated strains were recorded (Table 1, Fig. 1). Cell morphology of the pure cultures recorded upon Gram staining is presented in table 2 and Fig. 1. Recording different aspects of colony and cellular morphological characters will help in morpho typing of bacteria and help in purification of the isolates.

The identity of all the bacterial strains isolated from the gut contents of flacherie infected worms were determined by sequencing the 16S rRNA gene and subsequent BLAST search analysis in EZ taxon. Identifying and cataloging bacteria in concurrence with the taxonomic standards is imperative in studies related to determining the bacterial diversity of any sample. Sequencing of the 16S rRNA gene is the most desired and extensively employed protocol for determining the identity of bacteria (Pontes *et al*., 2007). Researchers across the globe believe it to be an ideal candidate in studies associated with taxonomy of bacteria due to the copious features it possess which include their presence in all bacterial members, size, degree of functional conservation across time, ease it offers in design of primers based on the conserved sequences it posses and presence of variable region aiding in distinguishing across taxa (Woese, 1987; Drancourt and Raoult, 2005).

The closest phylogenetic neighbors of the isolated strains are presented in table 2. The bacteria isolated in the present work are phylogenetically related to *Staphylococcus*, *Paracoccus*, *Bacillus*, *Enterococcus* and *Enterobacter*. Species belonging to *Staphylococcus* and *Bacillus* are most encountered members from flacherie infected worms. This has been reported by other research groups in the past (Chitra *et al.*, 1975; Enomoto, 1987; Nataraju *et al*., 1999, 2002; Priyadarshini *et al*., 2008). Members of *Enterococcus and Enterobacter* are reported to be the native gut microbiota in silkworm (Thirupathaiah *et al*., 2019). Thirupathaiah *et al*., 2019 states that gut symbionts belonging to *Enterobacter* aid in digestion of cellulose derived from mulberry as genetic constitution of *Bombyx mori* doesn't encode for cellulase. However, a member of the genus *Enterobacter* (*Enterobacter cloacae*) to be pathogenic to silkworm is also documented by Ayoade, 2014. Reports on neither *Paracoccus* being infective nor a native inhabitant of silkworm are scanty.

The pathogenicity of all the strains isolated in the present work was evaluated by inoculating the same (individually) to healthy silkworms on the first day of the III instar. Mortality due to bacteriosis from an

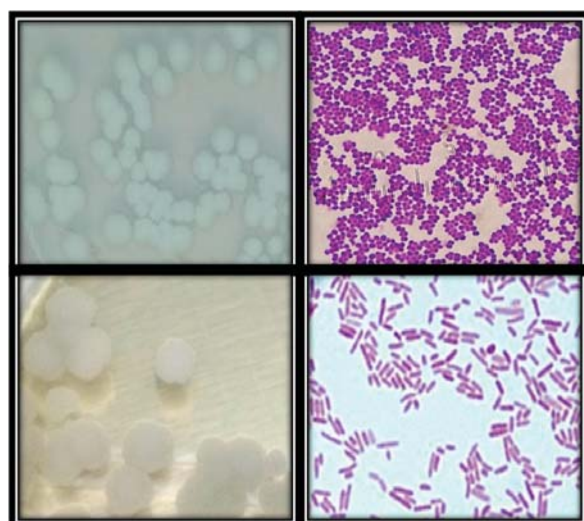

Fig. 1: Colony and cellular morphologies of *Staphylococcus argenteus* Bac1(top row) and *Bacillus carboniphilus* Bac3 (bottom row)

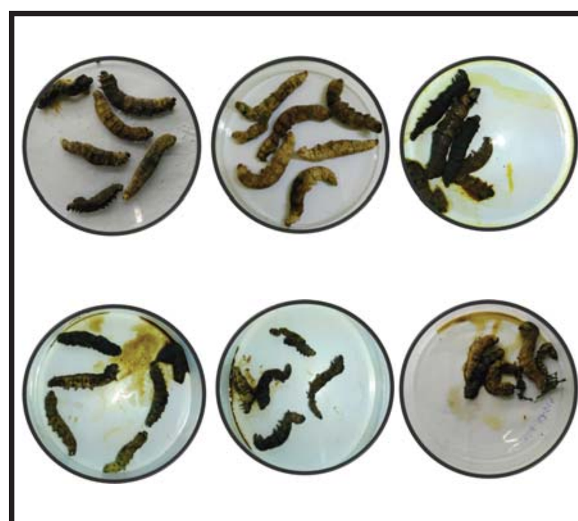

Fig. 2: Flacherie infected worms post inoculation. Flacherie infected worms from batches treated with Strain Bac1 (top row) & Bac3 (bottom row)

Table 1: Colony morphology of the bacterial strains

Strain	Colour	Shape	Elevation	Margin	Size	Texture	Pigmentation
Bac1	White	Irregular	Raised	Undulate	Moderate	Smooth	Non Pigmented
Bac2	Yellow	Round	Flat	Entire	Moderate	Smooth	Pigmented
Bac3	White	Round	Flat	Entire	Large	Slimy	Non Pigmented
Bac4	White	Round	Raised	Entire	Punctiform	Smooth	Non Pigmented
Bac5	White	Round	Flat	Entire	Small	Rough	Non Pigmented

Table 2: Cell morphology, motility, Gram stain character of the bacterial isolates and their closest phylogentic neighbours

Strain	Cell morphology	Motility (+/-)	Gram staining (+ve/-ve)	16S rRNA gene sequence similarity (%) with the nearest type strain	EMBL accession no.
Bac1	Cocci in clusters	-	Gram +ve	99.2% - *Staphylococcus argenteus* MSHR1132^T	LR736235
Bac2	Cocci	-	Gram -ve	100% - *Paracoccus niistensis* NII-0918^T	LR736236
Bac3	Rods	+	Gram +ve	100% - *Bacillus carboniphilus* Kasumi6^T	LR736237
Bac4	Cocci in clusters	-	Gram +ve	99.6% - *Enterococcus cecorum* A60^T	LR736238
Bac5	Rods	-	Gram -ve	98.9% - *Enterobacter asburiae* 1497-78^T	LR736239

Table 3: Pathogenicity of the isolated bacterial strains

Strain	Mortality (%)
Bac1	47.33 (43.45) ± 1.67*
Bac2	4.67 (12.41) ± 0.88
Bac3	56.67 (48.82) ± 1.021*
Bac4	6.67 (14.92) ± 0.75
Bac5	7.33 (15.59) ± 1.418
Control	6.67 (14.92) ± 0.75
SEm (±)	**1.024**
LSD (0.01)	**3.269**

*Note : * significant at 1% level; ( ) indicates Sin transformed values; ± standard error*

experimental bioassay conducted during July-August, 2019 is presented in table 3. As also evident from the past research studies (Chitra *et al.*, 1975; Enomoto, 1987; Nataraju *et al.*, 1999, 2002; Priyadarshini *et al.*, 2008), strains belonging to the genera *Staphylococcus* and *Bacillus* are inflicting ~50% mortality. Flacherie diseased larval samples (Fig. 2) were collected from the treatment batches individually and bacterial strains were re-isolated from the gut contents. *Paracoccus niistensis* Bac2, *Enterococcus cecorum* Bac4 and *Enterobacter asburiae* Bac5 couldn't be re-isolated from the flacherie infected worms of the respective batches and instead Gram positive cocci similar to *Staphylococcus* was re-isolated where as *Staphylococcus argenteus* Bac1 and *Bacillus*

carboniphilus Bac3 could be successfully re-isolated from the flacherie infected worms from the respective treatment batches, thereby establishing their pathogenicity.

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