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Molecular Identification and Pathogencity of Bacillus thuringensis SW2 on Silkworm (Bombyx mori L.)

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ABSTRACT : ***The silkworm is an important economic insect due to production of silk. It is the larva or caterpillar of the domesticated silk moth, (Bombyxmori L.) . In the rearing of silkworm bacterial flacherie considered the most important disease effect in silk production. Flacherie is a Syndrome associated with bacterial disease. In the present study the isolation of pathogenic bacteria from infected larvae . The infected larvae samples were serially diluted from which 10-5,10-6 and cultivated onto nutrient agar plate. The dominant colonies were selected identification by colony morphology, Gram staining property and biochemical test and confirmed the identification by 16SrRNA which proved the name of bacteria as Bacillus thuringensis SW2 with accession number (MK327364.1). The genus of Bacillus thuringensisSW2 was responsible of mortality in 4th and 5th instars of the larval stages of B. mori L. after 3 days recorded 4.66a and 2.66b in 4th, 5th instars respectively post infection and highest mortality rate was obtained at 5 days recorded 12a, 12.66a in 4th and 5thinstars receptively and the total injury reach to 31a,32a in 4th and 5thinstars receptively after 5days .***

KEYWORDS Silkworm, Bacillus thuringensis SW2, Bacterial Flacherie

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I. INTRODUCTION

The silkworm is the larva or caterpillar of domesticated silkmoth, BombyxmoriL.belong to family Bombycidae. silkworm is an important economic insect because of the commercial value of its silk . silkworm like white mulberry leaves in feeding , but also eat any other mulberry trees. It is entirely dependent on humans for its reproduction and no longer occurs naturally in the wild (Shan Wu et al .2010 andMeeramaideenetal., 2017). Silkworm diseases are considered the direct cause for the sericulture damage (Ponnuveletal., 2003). Bacterial and viral infections cause severe diseases in B. mori larvae and create a serious loss to silk industry (Raoetal., 2011).

Flacherie disease: is a syndrome of bacterial diseases, caused by an virus which is an exciting agent, followed by secondary infection of bacteria.

SYMPTOMS: infected larvae become lethargic and non-motile. The colour of the haemo-lymph converted to black. Sealing of anal lips, rectal protrusion, all symptoms are easily detected of the disease. infected larvae will die in short time, (Govindhanet al., 1998).

Among the protozoan , bacterial, viral and fungal pathogen, bacterial infection is more dominant in the silkworm ,Bombyxmori and the genus are linked to spread disease in B.mori during rearing majorly belongs to the guns Bacillus sp. such as Bacillusthuringiensis. the other symptoms include bacterial flacherie loss appetite, sluggishness of worms with slow growth, shrinkage, swelling of thorax, appearance of brown speeks on skin, straightened appearance of body, oral and anal discharge, liquefaction of inner organs, rupturing of skin and oozing out of oulsmelling brown liquid (Balavenkatasubbaiah, 2015) in the present study the isolation and identification of pathogenic bacteria with traditional methods molecular identification by 16SrRNA. The pathogencity test of B. thuringensis SW2was applied on healthy worm to study the rate of infection and developed symptoms.

II. MATERIALS AND METHODS

The present study carried out atPlant Protection Research Institute , Sericulture Department, Sharkia Branch.

1- Collection of sample and isolation of bacteria

Collect the diseased silkworm during rearing season may 2017, the samples put in sterilizedslain solution and dilute to 10-5- 10-6then plated onto nutrient agar plates. the plates incubated for 24hrs at 370C.according to (Aneja, 2003). Nutrient agar- medium (Oxoid Ltd., England): g/l Peptone ,5; Yeast extract ,2 Lab-Lemcopowder,1 ;Sodium chloride,5 Agar ,15; Distilled water,1000 ml

2- Morphological Identification&Gram stain and Biochemical tests used for identification of bacterial isolates.

Grams stain and biochemical test were applied for identification of isolated bacteria according to (Aneja, 2003).

3- Molecular identification of Bacillus thuringensis

Bacterial identification was based on 16S rRNA gene sequencing analysis and biochemical analysis using the QIAamp DNA Mini KitCatalogue no.51304. DNA extraction of bacterial isolate pellet was carried out according to QLAampDNAmini kit instructions.The purified DNA immediately was amplified by PCR using PCR Master Mix according to Emerald AMPGT (Takara RR310KIT) with recommend thermal cycling conditions (Acivation 940c for 15 min and 35 cycles of 940c for 30sec.,560c for 1min, 720c for 1min and 30 sec,extansion 720c for 10min)with the primers 16S-27 (5'-AGAGTTTGATCMTGGCTCAG- 3') and 16S-1492 (5'-TACGGYTACCTTGTTACGACTT-3')according to (Lagacé et al., 2004). The resulting PCR product was purified and stored at -200c according to (Sambrook et al., 1989). A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of LasergeneDNAStar software Pairwise, which was designed by Thompson et al., 1994) and Phylogenetic analyses were done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

4- Silkworm rearing

Silkworm larvae were reared on mulberry leaves at 27°,70% relative humidity , and 12h light :12h dark photoperiod rearing method (Krishnaswamy, 1979). while larvae reaching the 4th and 5th instars stage worms were infected and the sample were collected for further experiments.

6-The biological & technological parameters measured in experiments as follows

1- Biological studies
   A. Total injury (%)
   B. Cocooning percentage(%)

Cocooning percentage= no.of fresh cocoons/total no.of larvae at the 5th instar*100 (Krishnaswami, 1978).

Data obtained statistically analyzed according to Snedecor and Cochran (1982) methods using software Costat program2005) version 6.311.

5- Pathogenicity test of isolated bacteria on healthy silkworm:

Eggs of silkworm strain were obtained from the Plant Protection Research Institute , sericulture research

department, Egypt. started in 2nd May until 16 June 2019. The egg were incubated at 260C until hatching then reared by feeding on mulberry leaves in separate three groups, two groups in 4th and 5th instars infected with B. thuringensisSW2at 0.5 McFarland (cell density of 1.5x 106 cell/ ml was adjusted using saline) and used for silk worm oral infection spread on mulberry leaves nutrition and the third group for control (healthy) under the rearing condition of temperature 26+-30C with 75+-5% humidity after 1, 2, 3, 4 and 5 days. Young silkworm larvae fed on mulberry leaves until the molted to the third instars (Zannoon et al. 2008).After molted to their 4thand 5th instars, 50 larva were selected each for three replications for feeding on mulberry leaves spread with spore suspension of BT.SW2, larval left feeding for one meal first day in 4th and 5th instars then supplies with untreated leaves and the same symptoms developed during rearing. The control groups consisting of 50 larva in each of three groups were fed with natural mulberry leaves. After application, larval mortality percentages were daily recorded for 5 days of inspection. Mortality data were corrected according to Abbott'sformula (1925) as follows:

$$\text{Corrected mortality \%} = \frac{\text{Control mortality} - \text{Observed mortality}}{Control\ mortality} \times 100$$

The treated group with pathogenic bacteria as well as the control group were estimated of quality parameters like mortality of larvae in 4th and 5thinstars, Total injury and cocooning ratio percentage.

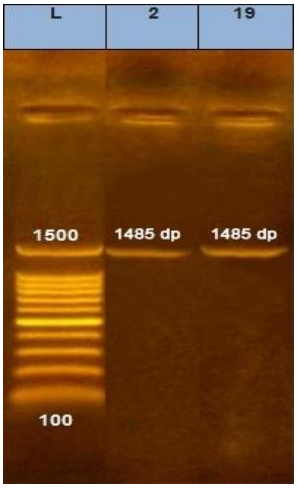
III. Results and Discussion

The predominant colonies from twenty bacterial isolate were identified by various biochemical and Gram stain recorded Bacillus spp., the predominant bacterial strains were isolated from infected larva (table 1) Bacillus thuringiensis SW2 which the main causative pathogen causes bacterial flacherie for silkworm so we confirm these isolate by 16SrRNA. Bacillus thuringiensiss SW2 16S ribosomal RNA gene, with accession no. MK327364.1 The physiological weakness in silkworm make them susceptible to pathogenic microbe such as different bacteria (Staphylococcus sp.,/Bacillus thuringiensis/ E.coli/Streptococcus sp., and Serratia marcescence) caused Bacterial Flacherie and non occluded viruses (BmIFV/BmDNV) Cause Viral Flacherie (Balavenkatasubbaiah & Sivaprasad 2015).

Sakthivel et al., (2012) reported that bacterial diseases are common in silkworms and massive out-breaks are frequent in the hot and humid summer and autumn rearing seasons, a group of bacteria causes infection leads to the flaccid of larvae which is termed as "Flacherie".

Table no.1Morphological and biochemical identification of isolated bacteria.

Test	Isolate no.2
Gram	+ve
Cell form	Rod with crystal
Hemolysis	+ve
Catalase	+ve
Oxidase	+ve
Urease	+ve
Citrate	+ve
Lactose	-ve
Maltose	+ve
Strach	+ve
Indol	-ve
H2s	-ve
Spore	+ve



(Fig. 1): Agarose gel electrophoresis of 16SrDNA gene. Lanes:

(Fig.2): gene tree of

(**Fig.3**) symptoms of infection by *Bacillus thuringensis* sw2

Pathogenecity of Bacillus thuringensis SW2

Feeding larvae in 4thand 5thof Bombyxmori with B. thuringensisSW2 reduced feeding activity, the vomiting and gradual shrinking of larvae with the progression of disease were the symptoms (Fig.3), showed infected larvae became lethargic, motionless, the colour turns to black and sealing were developed after 3 days post infection. Mortality attributable to infection occurred in 4th and 5th at about third day with mortality % 4.66+ 0.66 increased to 6.66+ 1.17 and 12+ 1.55 in fourth and fifth day respectively in 4th instars and in 5thinstars recorded 2.66+0.66 in third day increase to 6+1.15 and 12.66+2.66 in fourth and fifth day respectively post infection. The larval mortality percentage showed that highly significant between control and 5th instars as in (Table 2 and Fig. 4). The total injury percentage (Table 2) clear that feeding with contaminated with Bacillus thuringensisSW2 with 0.5 McFarland standards causes mortality in larvae as recorded in 4th instars 31a+1.33 and 32a+1.15 in 5th compared with control which recorded 12.6b+1.33. The cocoon percentages were recorded

87.33a+ 1.33 for control, indicated that caused reduction in cocoon percentages in 4th and 5thinstars were recorded significant between control and 5th instars which recorded 68b+ 1.15 as in (Fig.4).

Rearing condition is followed by mulberry leaves of poor quality (Manimegalaiandchandramohan 2005). The leaves of poor nutritive value will not be able to provide suitable quality of essential requirement to the larva to produce antibacterial factor, which result in high rate of multiplication of infectious bacteria and development of bacterial flacherie (Natraju et al.,2005). The etiological agent of bacterial flacherie had reported that was bacteria such as Staphylococcous aureus, Escherichia coli and Bacillus thuringiensisin silkworm (Bombyx mori L.) according to (Chitra et al., 1973).In these study we identified Bacillus thuriengesis SW2 this results were supported by(Anitha et al., 1994 and Sakthivel et al.; 2012). The major fact responsible for bacterial flacherie was the rearing conditions. the rise in temperature and humidity in rearing place leads to dysfunction of alimentary canal which increase flacherie. (Nataraju et al., 2005).

Table 2: Effect of mulberry leaves fortified with spore suspension of Bacillus thuringensis SW2 on biological activity of silkworm (Bombyx mori L.)

Treatment	Mortality % per day					Total injury	Cocoon%
	1	2	3	4	5		
Control	0	0	0c	0.66b+0.66	0b	12.6b+1.33	87.33a +1.33
4th larvae	0	0	4.66a+ 0.66	6.66a+1.76	12a+1.15	31a+1.33	68.6b +1.33
5thlarvae	0	0	2.66b+0.66	6a+1.15	12.66a+2.66	32a+1.15	68b +1.15
L.S.D.	0	0	1.88	4.41	5.80	4.41	4.41
P value	0	0	0.0027**	0.0302*	0.0029**	0.0001***	0.0001***

Data expressed as Mean + S.E. ***=p≤0.01

Mean under each variety having different letters in the same column denote a significant different (p≤0.05)

IV.CONCLUSION

Results showed that bacteria causes mortality, reduction cocoon percentage and total injury. The predominant isolates are Gram +ve bacteria and the major strain is Bacillus spp. were identified by 16SrRNA as Bacillus thuringensis SW2 which causes reduction in rearing .

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