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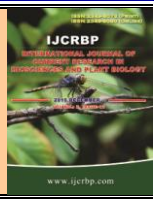

Original Research Article

Isolation and Identification of Some Bacteria Causing Infections in Silkworm (*Bombyx mori* L.)

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Abstract

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is a domestic economic insect for many people in different countries all over the world. It is very sensitive to different infection with many pathogens especially bacteria which accounts considerable loss of 10-15% to silk production. The goal of this investigation was to isolate and identify bacteria found in natural infection associated with external and internal fourth and fifth larval instars of *Bombyx mori*. These larvae were subjected to external and internal standard microbiological procedures of isolation. The identification of isolates was done using cultural, morphological, physiological and biochemical characteristics. A total of 14 isolates were successfully isolated from the outer body surface and nine isolates from the intestine of fourth and fifth instar silkworm larvae. The bacterial strains isolated from the infected larvae in this study were identified as follows: *Aeromonas sp*., *Paenibacillus macerans* (*Bacillus macerans*), *Bacillus megaterium, Bacillus licheniformis* and *Bacillus circulans*.

Keywords

Bambyx mori

Bacterial isolates

Silkworm

Introduction

Bombyx mori silkworm does not exist in the wild, being totally domesticated so excessive inbreeding has lowered drastically the immune factors in this insect, making susceptibility to diseases. So, the sericulture losses in the world mostly attributed directly to silkworm diseases, where these diseases occur due to various biological, physical, chemical, nutritional and environmental causes. Among these diseases, bacterial diseases which are mostly common, but in general massive out-breaks are rare. From the most serious bacterial diseases of silkworms, the lecheries which cause the silkworm cadavers to lose elasticity, soft, rapidly rot, swelling of thorax, appearance of brown sacks on skin, oral and anal discharges (Chakrabarty et al., 2012). In addition to the itemized symptoms, there is also a documented relationship between the occurrence of runts in silkworm's batches and infection of silkworm by bacterial pathogens (Singh et al., 2011). This disease can be classified into three groups, namely, bacterial toxicosis, bacterial gastroenteric disease and septicemia (Lu 1991). Also, silkworms are more sensitive to range of other bacterial pathogens including the following

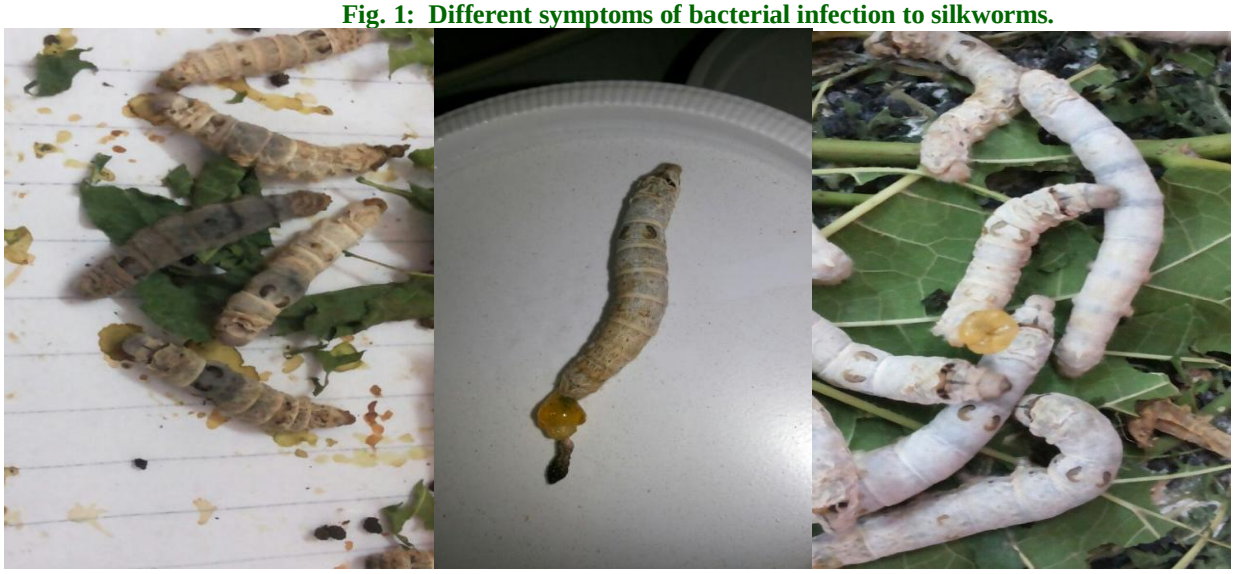
genera: *Bacillus*, *Serratia*, *Streptococcus*, *Staphylococcus*, *Enterobacter*, *Aeromonas and Pseudomonas*. The virulence of these bacteria to silkworm differ and course of disease is not uniform (Jiao et al., 2013).

Often rely isolation methods and identify the bacteria associated with the insects on the bacteria within the intestines only (Wei et al., 2011), or within the outer body surface, gut and whole body of insect (Chen and Huang 2008 and Femi et al., 2014).

During the sericulture of the insect we noticed that, the dead or moribund diseased from fourth or fifth larval instar have abnormal symptoms such as change in color into brown and black, cessation of feeding, loss of body luster, flaccidity, sluggishness and exiting of fluid from the anal lip and rectal protrusion (Fig. 1). These larvae were removed and stored in sterilized tightly closed vials at 4°C in refrigerator until they had been needed for complete the isolation and identification techniques for associated bacteria.

So, in the present investigation an attempt was made to isolate, characterize, describe and identify unknown bacteria associated with fourth and fifth larval instars of silkworm, using biochemical, morphological and physiological tests.

Fig. 1: Different symptoms of bacterial infection to silkworms.



Materials and methods

Collection of larvae

Healthy and diseased fourth and fifth larval instars of silkworms, *B. mori* were collected from Sericulture Laboratory of the Plant Protection Department, Faculty of Agriculture, Suez Canal then preserved in aseptic plastic containers and transported to Microbiology Department, Faculty of Agriculture, Fayoum University to complete the isolation and identification of associated bacteria with silkworm larvae.

Isolation techniques of associated bacteria

Bacteria associated with the above-mentioned larvae of the fourth and fifth instars of silkworm were isolated according to two main techniques:-

Outer body surface method

In order to reveal any bacteria associated with the subjected larvae, each of the refrigerated individuals was examined through 24-72 h from the time of storage under aseptic conditions where the larvae were sterilized by dipping in 2% sodium hypochloride (10% commercial solution) for 3-5 min, then passed through five separate washing with sterile distilled water (Campbell and Rodgwaite, 1971 and Femi et al., 2014). For insuring the appropriate surface sterilization, checks were made by spreading the washing solution on nutrient agar medium using the spread plate method and incubated at 30°C. Sterilized larvae were dried between two sterilized filter papers, cultured on nutrient agar medium in Petri-dishes of 9 cm. diameter, then incubated at 37°C for 24 h. Incubated dishes were daily observed inspected bacterial colonies which purified and stored on slants of EPPXOPRBTE media at 4°C and

cultured periodically and used for the subsequent experiments. Healthy silkworms larvae were subjected to the same procedures of isolation for the expected dormancy of bacteria.

Gridding method

Cadaver that has been previously surface- sterilized was ground with mortar and pestle in 10 ml sterile saline water there aliquot of the suspension was taken for serial dilutions up to 10^{-6}. 0point one ml of the suspension was inoculated onto nutrient agar media and incubated at 37°C for 48 h then the growing cultures were examined for the presence of different bacterial colonies. Distinct colonies were subsequently isolated for further processing (Nataraju et al., 2005 and Ganesh, 2013). The isolates obtained from the above mentioned two procedures were coded from which such isolates were obtained.

Identification tests

Morphological, biochemical and physiological characterization tests were carried out to identify the bacterial isolates as given hereunder.

Bacterial cells from typical colonies were scrapped after observation with a light microscope from the agar surface and suspended in sterilize tap water. The morphological characteristics of these isolates were limited to colony shape and properties, cellular morphology, Gram staining, spore formation and motility. The physiological-biochemical characteristics were oxidase, catalase, Voges-Proskauer, methyl red, indole, glucose fermentation test, hydrogen sulfide production, and hydrolysis of starch (Dong and Cai 2001). Also enzyme activities were investigated, using the API-ZYM system (API BióMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Finally, the isolates were identified according to Bergey's Manual of Systematic Bacteriology (Holt, 2005) and by use API 20N Kit and Carbohydrates fermentation patterns API 50 CHB Kit (Biomèrieux, Marcy l´Etoile, France) for complete the identification process of gram negative and gram positive isolates, respectively.

Results and discussion

Isolation of bacteria

Results of isolation methods described above gave total of 14 isolates which successfully collected from the outer body surface and nine isolates were collected from the intestine of fourth and fifth instar silkworm larvae. These isolates were classified into five phenotypes based on the colony shape and properties, cellular characteristics, gram stain, spore- formation and motility. they were given code isolates the first two characters in the silkworm next to the serial number SW1, SW2, SW3, SW4 and SW5. Results of morphological characterization of bacterial isolates are presented in Table 1showed that, SW2, SW3, SW4 and SW5 isolates were G+ bacteria while the isolate SW1 was G- bacteria. SW1, SW2 and SW5 isolates were facultative anaerobic while SW3 and SW4 isolates were aerobic. The isolates SW1, SW2, SW4 and SW5 were motile while isolate SW3 was not, respectively.

Table 1. Morphological characterization of bacterial isolates from *B. mori*.

Bacterial isolate	Colony shape and properties	Cellular characteristics	Oxygen requirement	Gram stain	Spore formation	Motility
SW1	Punctiform, Pink, Slime	Short Rod	Facultative anaerobic	-	-	+
SW2	Irregular, White, Rough	Rod	Facultative anaerobic	+	+	+
SW3	Circular, Orange, Slime	Rod	Aerobic	+	+	-
SW4	Circular, White , Buttery	Rod	Aerobic	+	+	+
SW5	Punctiform, Bright red, Cream	Rod	Facultative anaerobic	+	+	+

Biochemical and physiological characterization of bacterial isolates

Results of the biochemical and physiological characterization of isolates are presented in Table 2. The isolates were found to diverseand differs in ability of different isolates fermentation of sugars. While similar in ability to utilize-Arabinose, Trehalose, Inulin and Glycogen. All isolates produced peroxidase and were negative for the Voges-Proskauertest, L-Arabinose, Inulin and α-fucosidase. Positive for the production of esterase (C4), Esterase Lipase (C 8) and Trypsin: the isolated bacteria fermented Esculin, Mannitol, Sorbitol and Lactose (Table 2).

Table 2: Physiological and biochemical characteristics of bacterial isolates from *B. mori*.

No.	Characteristics	Bacterial isolates				
		SW1	SW2	SW3	SW4	SW5
1	Oxidase	-	+	-	-	-
2	Catalase	+	+	+	+	+
3	Vogas-Proskauar	-	-	-	-	-
4	Hippurate	+	+	+	+	+
5	Esculin	+	+	+	+	+
6	Pyrrolidonyl Arylamidase	-	+	-	+	-
7	Alkaline Phosphatase	-	+	-	+	-
8	Leucinearylamidase	-	-	-	-	-
9	Arginine dihydrolase	+	+	+	+	+
10	Ribose	-	+	-	+	+
11	L-Arabinose	-	-	-	-	-
12	Mannitol	+	+	+	+	+
13	Sorbitol	+	+	+	+	-
14	Lactose	+	+	+	+	+
15	Trehalose	-	-	-	-	+
16	Inulin	-	-	-	-	-
17	Raffinose	-	+	-	+	+
18	Starch	+	-	+	-	-
19	Glycogen	-	-	-	-	+
20	Alkaline phosphatase	+	-	+	-	+
21	Esterase (C4)	+	+	+	+	+
22	Esterase Lipase (C 8)	+	+	+	+	+
23	Lipase (C14)	+	-	+	+	+
25	Valinearylamidase	+	+	+	+	+
26	Cystinearylamidase	+	+	+	+	+
27	Trypsin	+	+	+	+	+
28	α-chymotrypsin	+	+	+	+	-
29	Acid phosphatase	-	-	+	+	+
30	Naphthol-AS-Bl-phosphohydrolases	+	+	+	+	+
31	α-galactosidase	-	+	-	+	+
32	β-galactosidase	-	+	-	+	+
33	β-glucuronidase	-	-	-	-	+
34	α-glucosidase	-	-	-	-	+
35	β-glucosidase	-	+	-	-	-
36	N-acetyl-β-glucosaminidase	-	-	-	-	+
37	α-mannosidase	+	-	-	-	-
38	α-fucosidase	-	-	-	-	-

Table 3. Carbohydrate fermentation patterns of selected isolates by API 50 CHB system.

No.	Carbohydrates	Bacterial isolates				
		SW1	SW2	SW3	SW4	SW5
0	Control	-	-	-	-	-
1	Glycerol	+	+	-	+	+
2	Erythritol	-	-	-	-	-
3	D-Arabinose	-	-	-	-	-
4	L-Arabinose	+	+	-	+	+
5	D-Ribose	+	+	-	+	+
6	D-Xylose	-	+	-	+	+
7	L-Xylose	-	-	-	-	-
8	D-Aonitol	-	-	-	-	-
9	Methyl-β-D-Xylopyranoside	-	+	-	-	-
10	D-Galactose	-	+	-	+	+
11	D-Glucose	+	+	-	+	+
12	D-Fructose	-	+	+	-	+
13	D-Manose	-	+	-	+	+
14	L-Sorbose	-	-	-	-	-
15	L-Rhamnose	-	+	-	-	-
16	Dulciol	-	-	-	-	-
17	Inositol	+	-	-	-	-
18	D-Mannitol	-	+	-	+	+
19	D-Sorbitol	-	-	-	+	+
20	Methyl-α D-Mannopyranoside	-	-	-	-	-
21	Methyl-α Glucopyranoside	+	+	-	+	+
22	N-Acetyl-Glucosamine	-	-	-	+	+
23	Amygdalin	+	+	-	+	+
24	Arbutin	-	+	+	+	+
25	Esculin Ferric Citrate	+	+	-	+	+
26	Salicin	+	+	-	+	+
27	D-Cellobiose	+	+	-	+	+
28	D-Maltose	+	+	-	+	+
29	D-Lactose	+	+	-	+	+
30	D-Melibiose	-	+	-	-	+
31	D-Saccharose (sucrose)	+	+	+	+	+
32	D-Trehalose	+	+	-	+	+
33	Inulin	-	-	-	-	-
34	D-Melezitose	-	+	-	+	+
35	D-Raffinose	+	+	-	+	+
36	Amidon (Starch)	+	+	+	+	-
37	Glycogen	+	+	-	-	+
38	Xylitol	-	-	-	-	+
39	Gentibiose	-	+	-	+	+
40	D-Turanose	-	+	-	-	-
41	D-Lyxose	-	-	-	-	-
42	D-Tagtose	-	-	-	+	+
43	D-Fucose	-	-	-	-	-
44	L-Fucose	-	-	-	-	-
45	D-Arabitol	-	-	-	-	-
46	L-Arabitol	-	-	-	-	-
47	Potassium Gluconate	-	-	-	+	+
48	Potassium 2-Keto Gluconate	-	-	-	-	-
49	Potassium 5-Keto Gluconate	-	-	-	-	-

Identification of bacterial isolates

Depending on biochemical, physiological and morphological characteristics of the bacterial isolates (Tables 1and 2), using API 20N Kit and Carbohydrate fermentation patterns API 50 CHB Kit (Biomèrieux, Marcy l´Etoile, France) for identification of gram negative and gram positive isolates, respectively (Table3). As shown in Table 3, five bacterial isolates 14 isolates were identified as follow:- SW1*Aeromonas sp*., SW2*Paenibacillus macerans* (*Bacillus macerans*), SW3 *Bacillus megaterium*, SW4 *Bacillus licheniformis* and SW5 *Bacillus circulans*, respectively. These results were supported by Anitha et al. (1994) and Sakthivel et al. (2012).

The major factor responsible for bacterial infection may be the rearing conditions such as temperature and humidity. The rising in temperature and humidity in rearing place leads to dysfunction of alimentary canal which encourages bacterial infection (Nataraju et al., 2005). The leaves of poor nutritive value will not be able to provide sufficient quality of essential requirements to the larva to produce antibacterial factor, which results in high rate of multiplication of infectious bacteria and development of bacterial diseases.

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