Research Article

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

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Abstract: Totally, 82 samples from air and various surfaces of shelves (wood), grids (metal), wall (wallpaper) and floor (linoleum) in a silkworm rearing room were taken and analysed. Twenty two bacterial isolates were identified as Gram-positive: spore-forming microorganisms, staphylococci and streptococci. According to biochemical identification of isolates by Micronaut-Scan, we confirmed isolates as *Paenibacillus macerans*, *Bacillus sphaericus*, *Bacillus circulans*, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus* spp. Susceptibility of some selected isolates to various drugs was tested. Resistance to important groups of antibacterials (penicillin, chloramphenicol, quinolones, macrolides, tetracyclines and sulfonamides) was detected. Contamination of various surfaces with these bacteria is a potential risk of infection in silkworm.

Key words: silkworm, *Bombyx mori*, bacteria, drug resistance

Introduction

Silkworm diseases are due to various biological, chemical, physical, nutritional and environmental causes. The silkworm *Bombyx mori* L. does not exist in the wild and exists only as domesticated. Excessive inbreeding has lowered drastically the immune factors in this group of insects, making them particularly susceptible to diseases and changes in the environment. Globally, silk of *Bombyx mori* remains the most highly demanded, with up to 95% demand rate. Major constraint to *Bombyx mori* silkworm production is the scourge of pathogenic diseases. A code of conduct for rearing silkworm is practiced to ensure survival of silkworm and cocooning.

Bacterial diseases such as bacterial septicaemia, bacterial toxicities and bacterial gastro-enteric diseases have also been reported in the silkworm. Bacteria that induce flacherie include *Bacillus* spp., *Streptococcus* spp., *Staphylococcus* spp**.,** *Streptococcus faecalis*, *Streptococcus liquifacions*, *Staphylococcus acire*, *Staphylococcus epidermidis*, *Bacillus thuringiensis*, *Serratia marcescens, Micrococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Aerobacter cloacae*, *Achromobacter delmarvae* and others (Karthikairaj *et al*. 2013). Symptoms of flacherie include loss of appetite, sluggishness of worms with slow growth, shrinkage, swelling of thorax, appearance of brown specks on skin, straightened appearance of body, oral and anal discharge, liquefaction of inner organs, rupturing of skin and oozing out of foul smelling brown liquid (Zhang *et al*. 2013). In addition to the itemized symptoms, there is also a documented relationship between the occurrence of runts in silkworm batches and infection of silkworm by bacterial pathogens. In most cases, the runts develop when infected insects' anal lips become sealed with a sticky soil-coloured semi-solid thereby preventing the insect from feeding and such individuals shrink lengthwise (Sahay *et al*. 2000, Singh *et al*. 2011).

Bombyx mori is an insect with a high socio-economic and cultural value. The present study aims to identify, characterize and describe the bacterial Gram-positive pathogens of the silkworm *Bombyx mori* in breeding room's surfaces in Bulgaria before disinfection. This is expected to create base line data for future breeding programs against silkworm diseases in our country.

Materials and Methods

The experiment was conducted during 2014 and 2015. The samples were collected from the experimental base of Agrarian Faculty, Trakia University, Stara Zagora, and bacteriological assay was carried out in NDRVMI – Sofia. From various surfaces (wood, metal, wall and floor) of the environment in the room for silkworm rearing before disinfection, we collected 82 samples (by swabs in physiological saline from 1 cm^2 area).

Morphological, biochemical and physiological characterization tests were carried out to identify the bacterial isolates as follows. Bacterial cells from typical colonies were scrapped after observation under a light microscope from the agar surface and suspended in sterilize purified 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 & Cai 2001). The isolates were confirmed by use of Micronaut bacteria identification system (Merlin, Germany). Antibiotic susceptibility tests included an assessment of the antibiotic resistance of isolates to different drugs performed by mean of Merlin Micronaut semi-automated system (Panel type R3).

Results and Discussion

Isolation of bacteria

Isolation methods gave a total of 22 isolates, which were successfully collected from different room surfaces for keeping and breeding of silkworm before disinfection. Results of morphological characterization of bacterial isolates are presented in Table 1. Based on their biochemical characteristics, all isolates were confirmed by Micronaut bacteria identification system. Isolation of *Paenibacillus macerans* from all tested surfaces was more often than isolation of *Bacillus sphaericus*, *Bacillus circulans*, *Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus*

Table 1. Morphological characterization of bacterial isolates from room surfaces for breeding of Bombyx mori

Surface	Bacterial isolate (n)	Colony shape and properties	Cellular characteristics	Oxygen requirement	Gram stain	Spore formation	Motility
Wood Metal Wall Floor	*P. macerans* (7)	Irregular, white, rough	Rod-shaped	Facultative anaerobic	+	+	+
Wood Metal	*B. sphaericus* (2)	Creamy	Rod -shaped	Obligate aerobe	+	+	+
Wood	*B. circulans* (1)	Creamy	Rod-shaped	Facultative anaerobic	+	+	+
Wood Metal	*B. cereus* (2)	Dull gray and opaque with a rough matted surface on blood agar	Large, 1 x 3-4 µm, rod-shaped	Facultative anaerobic	+	+	+
Wall Floor	*St. aureus* (2)	Large yellow colony on rich medium often hemolytic on blood agar	Cocci, arranged in grape-like clusters	Facultative anaerobic	+	-	-
Floor	*St. epidermidis* (1)	Small white colonies about 1–2 mm in diameter after overnight incubation, and is not hemolytic on blood agar	Cocci, arranged in grape-like clusters	Facultative anaerobic	+	-	-

Table 2. Biochemical characteristics of the most frequent isolated bacteria

Reactions	**P. macerans**	**B. cereus**	**P. macerans**	**P. macerans**	**P. macerans**	**B. sphaericus**	**P. macerans**	**P. macerans**	**B. sphaericus**	**P. macerans**
Peptidases:										
BZAR	+	+	+	+	+	+	+	+	+	+
APPA	+	-	+	+	+	+	+	+	+	+
LEU	-	+	+	+	+	-	+	+	+	+
PYR	-	-	-	-	-	-	+	-	-	-
D-ALA	+	-	-	+	-	-	+	+	+	-
ASP	-	-	-	-	+	-	-	-	-	+
GLY	+	-	+	+	+		+	-	+	+
PROL	+	-	+	+	+	-	+	+	+	+
L-ALA	-	-	+	+	-	-	+	-	-	-
GLYP	+	+	+	+	+	-	+	+	+	+
LYS	-	+	+	+	+	+	+	-	-	+
TYR	-	-	+	+	+	-	-	-	-	+
HPR	-	-	-	-	-	+	-	-	-	+
GLYT	-	-	-	-	-	+	-	-	+	+
ARG	-	+	+	+	+	+	+	+	+	+
Glucosidases/esterases:										
α- Glucosidase GLU	+	-	-	-	-	-	+	+	+	-
β- Glucosidase GLU	+	-	-	-	-	-	-	+	+	-
α- Galactosidase GAL	+		-	-	-	-	+	-	-	-
p-Nitrophenyl-β-galactosidase PNPG	-	-	-	-	-	-	-	-	-	-
α- N- Acetylglucosamidase α CHIT	-	-	-	-	-	-	-	-	-	-
Chitinase CHIT	-	-	+	+	+	+	-	+	+	+
p-Nitrophenyl-β-glucuronidase PGUR	-	-	-	-	-	-	-	-	-	-
α- mannosidase MAN	-	-	-	-	-	-	-	-	-	-
β- Fucosidase FUC	-	-	-	-	-	-	-	-	-	-
Diphosphoesterase DIP	+	+	+	+	-	+	+	+	+	+
β- Xylosidase PNPX	-	-	-	-	-	-	-	-	-	-
Phospholipase LIPC	+	+	+	+	+	+	+	+	+	+
Fermentations:										
GLUF	+	+	-	-	-	-	+	+	-	-
MANF	+	+	-	-	-	-	-	+	-	+
TREF	-	-	-	-	-	-	-	-	-	-
MATF	-	-	-	-	-	-	-	-	-	-
SORF	-	-	-	-	-	-	-	-	-	-
RAFF	-	-	-	-	-	-	-	-	-	-
MALF	-	-	-	-	-	-	-	-	-	-
SUCF	-	-	-	-	-	-	-	-	-	-
INUF	-	-	-	-	-	-	-	-	-	-
LACF	-	-	-	-	-	-	-	-	-	-
RIBF	-	-	-	-	-	-	-	-	-	-
XYLF	-	-	-	-	-	-	-	-	-	-
TAGF	-	-	-	-	-	-	-	-	-	-
TURF	-	+	-	-	-	-	-	-	-	-
Decarbixylases:										
URE	-	+	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-
ADH	-	+	-	-	-	-	-	-	-	-

Table 3. Antibiotic susceptibility of some Gram-positive bacterial isolates

Antibiotic	*Staphilococcus aureus/MIC*	*Streptococcus* spp*/MIC*	*Bacillus circulans/MIC*	*Bacillus cereus/MIC*	*Bacillus sphaericus/ MIC*	*Paenibacillus macerans/MIC*
AMC	I (=8/2)	I (=8)	S (<=0.125)	R (>8)	R (>8/2)	R (>8/2)
AMP	I (=4)	S (=2)	S (<=0.125)	R (>8)	R (>8)	R (>8)
APR	R (=32)	R (>32)	S (<=16)	S (<=16)	S (<=16)	S (<=16)
CEQ	R (>2)	R (>4)	S (<=2)	R (>4)	R (>2)	R (>2)
CET	I (=4)	S (<=2)	R (>4)	R (>4)	R (>4)	R (>4)
CMP	S (<=4)	S (<=2)	R (>4)	R (>4)	R (>8)	R (>8)
COL	R (>2)	R (>2)	S (<=1)	R (>2)	R (>2)	I (=2)
ENR	R (>1)	R (>2)	R (>2)	R (>2)	I (=1)	I (=1)
ERY	S (<=1)	R (>1)	S (<=0.5)	S (<=0.5)	R (>4)	R (>4)
FLL	S (<=2)	S (<=1)	S (<=1)	S (<=1)	I (=4)	R (>4)
GEN	R (>4)	S (<=2)	S (<=2)	S (<=2)	I (=4)	I (=4)
LIN	R (>4)	S (<=1)	S (<=1)	S (<=1)	R (>4)	R (>4)
LIS	R (>8/32)	R (>4)	R (>4)	R (>4)	R (>8/2)	R (>8/2)
MAF	R (>1)	S (<=4/16)	S (<=4/16)	S (<=4/16)	R (>1)	I (=1)
NEO	R (>16)	R (=16)	S (<=8)	S (<=8)	S (<=8)	R (=16)
PEN	R (>1)	R (>4)	R(>4)	R (>4)	R (>1)	R (>1)
SMO	R (>64)	I (=1)	S (<=0.125)	R (>1)	R (>64)	R (>64)
STR	R (>8)	R (>152)	S (<=38)	I (=152)	R (>8)	R (>8)
SXT	I (=64)	I (=4)	S (<=1)	I (=4)	R (>64)	R (>64)
TET	S (<=1)	S (<=16)	S (<=16)	S (<=16)	S (<=1)	I (=4)
TIA	R (>8)	I (=64)	S (<=16)	I (=64)	R (>8)	R (>8)
TILM	R (>16)	I (=4)	S (<=1)	I (=4)	R (>16)	R (>16)
TLS	R (>2)	R (>1)	S (<=1)	S (<=1)	R (>2)	R (>2)

epidermidis. In our previous studies in Bulgaria, we isolated *Bacillus cereus, B. thuringiensis* and *B. subtilis* (Gurgulova 1998, Vasileva 1998), which suggested that they were widespread in the premises for silkworm rearing.

Biochemical and physiological characterization of bacterial isolates

Results of the biochemical and physiological characterization of the most frequent isolated strains *Paenibacillus macerans*, *Bacillus cereus* and *Bacillus sphaericus* are presented in Table 2. The isolates were found to differ in ability of fermentation of glucose and mannose. While similar in disability to utilize arabinose, trehalose, inulin, sorbit, rafinose, maltose, sucrose, etc., all isolates of *P. macerans* produced phospholipase and were negative for the Voges-Proskauer test, urease, ornithine decarboxylase and arginine decarboxylase tests.

Identification of bacterial isolates

Depending on biochemical, physiological and morphological characteristics of all 22 bacterial isolates (Tables 1 and 2), Micronaut bacteria identification system, plate RPO for Gram-positive bacteria was used for identification of all isolates (Table 2). As shown in Table 1, bacterial isolates were identified as follow: 1. *Paenibacillus macerans* (*Bacillus macerans*) (n=7), 2. *Bacillus cereus* (n=2), 3. *Bacillus sphaericus* (n=2), 4. *Bacillus circulans* (n=1), 5. *Staphylococcus aureus* (n=2), 6. *Staphylococcus epidermidis* (n=1) and *Streptococcus* spp. (n=1). These results were in agreement with data reported by ANITHA *et al*. (1994), Sakthivel *et al*. (2012) and Adel *et al.* (2015). 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).

Antibiotic susceptibility test

According to the antibiotic susceptibility tests, all *Paenibacillus macerans* isolates were sensitive just to apramycin. Resistance was observed against erythromycin, florphenicol, cefquinome and many other antibiotics (Table 3).

Intermediate susceptibility was detected against enrofloxacin, marbofloxacin, gentamycin, tetracycline, etc. *Bacillus sphaericus* isolate was

susceptible just to three antibiotics: apramycin, neomycin and tetracycline. Resistance was observed to erythromycin and to the most of the other tested antibiotics. Intermediate susceptibility was detected to enrofloxacin, florphenicol and gentamycin. The most susceptible Gram-positive bacterial isolate was *Bacillus circulans*, which was susceptible to 18 antibiotics and resistant to 5 ones. The most resistant isolate was *Paenibacillus macerans,* which was resistant to 17 of the tested 23 antibiotics.

Previous studies reported isolation of bacteria of different genera from diseased silkworm, such as *Bacillus subtilis*, *Bacillus cereus, Staphylococcus albus, Staphylococcus aureus* (Priyadharshini *et al*. 2008) and *Streptococcus faecalis* (Patil 1994), *Bacillus thuringienisis* (Nataraju *et al*. 1991), *Streptococcus* spp. *Bacillus* spp. (Anitha *et al*. 1994). Antibiotics were used to find out their effectiveness against pathogenic bacteria (Mahmoud *et al*. 2012). As a result, bacteria associated with silkworm are prone to develop resistance to commonly used antibiotics. Twenty three antibiotics were used in this study and only three antibiotics, i.e. gentamycin, florpfenicol and apramycin, were shown strongly effective against most of the isolated bacteria (Table 3). This high antibiotic resistance of the isolates indicated reduced usability of current antibiotics. Similar antibiotic resistance of bacteria associated with flacherie has been reported by many previous studies (Nahar 1995, Kim *et al.* 2002). Antibiotics improve feed consumption and growth by stimulating metabolic processes within the silkworm as well as reduce the occurrence of diseases, which causes immense loss to sericulture industry. It has been found that ciprofloxacin significantly increases the effective rate of rearing and cocoon weights and cocoon length and width were significantly increased under the effects of antibiotic treatment comparing with control. Similar effect by gentamycin was reported by Mahmoud *et al*. (2012). Use of antibiotic to prevent bacterial disease of silkworm has also been reported by many studies (Hamamoto *et al*. 2005, Kaito *et al*. 2002). Administration of antibiotics and dose of administration are critical as in many cases administration of antibiotics was reported to have detrimental effects on intestinal microflora of silkworms, which caused adverse effects on the physiological system (Subramanian *et al*. 2009). As a result, it is recommended to apply low concentration of antibiotics to induce prophylactic measures to prevent bacterial infections, as also recommended by other researchers (Sheebha *et al*. 2008, Anandakumar *et al*. 2012).

Conclusion

A total of 22 isolates was successfully identified before disinfection from different room surfaces for keeping and breeding of silkworm. Isolation of *Paenibacillus macerans* from all tested surfaces was more often than isolation of *Bacillus spaericus, Bacillus circulans, Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus* spp.

Environmental Gram-positive bacteria, which were isolated from the room for silkworm breeding, can get into the body of silkworms in contact or by feeding and pose a potential danger to occurrence of disease. The major factors responsible for bacterial silkworm diseases are the rearing conditions. The rise in temperature and humidity in rearing place leads to dysfunction of alimentary canal, which encourages the bacterial diseases. It was found that isolated Gram-positive bacteria associated with silkworm are prone to develop resistance to commonly used antibiotics. Twenty three antibiotics were used in this study and only three antibiotics (tetracycline, florphenicol and apramycin) were shown strongly effective against most of the isolated bacteria. Resistance was observed against erythromycin, lincomycin/spectinomycin and cefquinome. The high antibiotic resistance of the isolates indicated reduced usability of current antibiotics. This finding highlighted importance of strict and effective disinfection of the room for silkworm rearing.

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