

Antibiotic (Gentamicin) Impact on Bacterial Flacherrie Disease of Silkworm, *Bombyx mori* L..

Souad M. Mahmoud, Rehab H. Taha and Saad, I. A. I.

Sericulture Department, Plant Protection Research Institute, Agricultural Research
Center, Egypt

ABSTRACT

There are many factors that influence the success of silk production. Bacterial flacherrie considered the most important factor. Antibiotics showed promising results for controlling of silkworm diseases and to improve the production of silk and harvesting superior crop. The effect of antibiotic (Gentamicin) upon the infection with bacterial flacherrie was discussed. Gentamicin as a cheap antibiotic in Egypt can be used easily by trainees and farmers. It was found that Gentamicin significantly increases the effective rate of rearing (ERR %) and larval weight (97.333% and 2.631gm, respectively). Single female and male cocoon weights (1.400 gm and 1.068 gm, respectively) and male cocoon shell ratio (22.708%) were significantly increased under the effect of antibiotic treatment comparing with control. Polymorphism among the tested haemolymphal samples were investigated using SDS-PAGE electrophoresis. A variation was detected in the protein banding pattern with considerable polymorphism (88.2 %), with the appearance of 2 monomorphic bands, 12 polymorphic and 3 unique bands.

Keywords : Antibiotic - Bacterial Flacherrie Disease - *Bombyx mori*

INTRODUCTION

Bombyx mori L. has an economic importance because of the commercial value of its silk. Therefore several trials for developing the biological processes and improving the qualitative and quantitative characters of silk yield took place (Taha, 2002).

Bacterial flacherrie is a common disease of mulberry silkworm. The aetiology of bacterial diseases is not fully understood because of the multiplicity of bacterial types involved in bacterial infections (Choudhury *et al.*, 2002). Generalized symptoms of insects associated with bacterial infections are loss of appetite, diarrhea (discharge of watery feces), and vomiting then the larvae softening and die emitting a foul odor (Tanada and Kaya, 1993). Since there are no specific preventive measures for the occurrence and spread of disease other than sanitized rearing methods, the only commercial practice today is to discard large stocks of worms in case of infection to avoid the spread of disease (Acharya *et al.*, 2002). Several prophylactic and curative measures are aimed for managing the disease taking into account the ecofriendly nature and cost effectiveness. Antibiotics are widely used in sericulture industry as a component of bed disinfectants and as therapeutic applications against bacterial diseases (Subramanian *et al.*, 2009).

In sericulture the productivity and quality largely depends on the healthiness, growth of the silkworm larvae and the suitable environmental conditions. Growth and development of larvae depends on the physiological processes that take place in the silkworm. Therefore, improvement of silk quality means improvement of the feed nutrition and upkeep of the larval health, since silkworm is highly susceptible to infection caused by major pathogenic groups. Broad spectrum antibiotics viz.,

penicillin, streptomycin, tetracycline and chloramphenicol were already tried on silkworm and found successful (Venkatesh and Srivastava, 2010). Antibiotics in silkworm are approved for four different purposes: disease treatment, disease prevention, disease control and for health maintenance or growth promotion (Phillips *et al.*, 2004).

Haemolymph has a key role in innate immunity response, which is triggered when bacteria enter the silkworm body (Hou *et al.*, 2010). Insects possess an effective innate immune system against foreign microorganisms. Innate immunity of insects is divided into two major reaction types: humoral and cellular reactions. Humoral reactions involve soluble proteins in the hemolymph such as phenoloxidase, antimicrobial proteins (AMPs), lysozymes, and lectins, whereas hemocytes mediate cellular reactions such as phagocytosis, encapsulation and nodule formation. In *Bombyx mori*, six different families of AMPs have been identified: Cecropin, Attacin, Lebocin, Moricin, Gloverin, and Defensin. One lysozyme and three lysozyme-like proteins, one of which is involved in elimination of invading pathogens, are also found in the silkworm (Tanaka and Yamakawa, 2011).

The present study is a trail for enhancing larval resistance against bacterial infection which is caused by several bacterial types by using a commercial antibiotic (Gentamicin) and its effect on biological and biochemical characters.

MATERIAL AND METHODS

Silkworm eggs of univoltine race, was obtained from the Sericulture Research Department (SRD) of Plant Protection Research Institute (PPRI).

Isolation of bacteria

Bacterial pathogens were collected from diseased black thorax Septicemia infected larvae, the diseased larvae were crushed by using mortar and pestle and the solution was filtered. The filtrate was centrifuged at 4000-5000 rpm for 10 minutes. The supernatant was discarded and the pellet was used for bacterial culture after re-suspending in distilled water (Aneja, 2003).

Preparation of Luria Agar (LA) medium and bacterial culture

After culture preparation, the bacterial sample was streaked in LA under aseptic conditions in a laminar air flow chamber with the help of streaking loop then incubated at 37°C overnight. After 24hr the bacterial growth was noticed, and further it was sub cultured. A sample of bacteria was taken with the help of a loop and centrifuged for 15 minutes at 4000 rpm. By discarding the supernatant, pellet sedimented at the bottom of the tube was dissolved in distilled water. The presence of bacteria was confirmed by staining with basic dyes like crystal violet and methylene blue. (Suparna *et al.*, 2011).

Infecting silkworm larvae with bacteria

The rearing technique was done in the lab at 28 ± 1 °C and $75 \pm 5\%$ RH, following the standard methodology of rearing according to (Krishnaswamy, 1978).

Larvae were fed on leaves of Kokuzo-27 mulberry variety. After the second moult, larvae were separated into three groups; control group, infected group and treated group with 50 larvae/ replica for each group.

Silkworms were infected with the bacteria by smearing the bacterial solution onto the leaf surface and dried in shade then fed to larvae. The second group were fed with distilled water smeared mulberry leaves and considered as a control. For the diseased and treated groups, the infected leaves were provided during the first feed on first day and thereafter the larvae were fed with normal leaves. On the next day, the fresh leaves dipped in Gentamicin (40 mg/1000 ml distal water) were fed to the

treated group only. The control and diseased groups were fed on normal leaves. Treatments were administered twice, once on the second day of the third instar and the other on the first day of the fourth instar (Manimegalai *et al.*, 2010).

Observations were made for each group and each replicate on larval duration, effective rate of rearing (ERR), larval weight, male and female cocoon weight, shell weight and shell ratio. Data were recorded and subjected to analysis of variance according to Snedecor and Cochran (1981).

At the sixth day of the fifth larval instar haemolymphal samples were collected from control group (healthy and naturally diseased larvae), infected group (healthy and diseased larvae) and treated group (healthy and diseased larvae); lanes 1, 2,3,4,5 and 6 respectively and stored in eppendorf tube.

Preparation of protein sample

10 microlitres of haemolymph sample was taken in an eppendorf tube which was collected from each group. 100 microlitres of Tris buffer (pH 6.8) was taken and mixed with haemolymph sample to dilute the sample, and from that 15 microlitres was taken in another eppendorf tube equal volume of sample buffer was also added.

The sample in the eppendorf tube was thoroughly mixed with the help of vortex. The sample solution was heated in boiling water for 2-3 min to ensure complete interaction between proteins and SDS, and a pinch of Bromophenol blue is used as a tracking dye.

Electrophoresis

Electrophoresis of sample solution was carried out in 12% separating gel. The gel run at 150V for 10-20 min until the tracking dye moved till the end of the gel. After run was completed the gel was removed and is stained with Coomassie Brilliant Blue for 45 min-1hr. Standard molecular weight markers were used for estimating the molecular weight (Laemmli, 1970).

RESULTS AND DISCUSSION

Bioassay:

The mean values of the most important economical characters are given in Table (1). Larval duration showed significant increase in infected group (1008, 888 and 816 hrs, respectively).

The highest effective rate of rearing (ERR %) and mature larval weight (gm) were recorded for the treated group (97.333 % and 2.631gm, respectively).

Single female and male cocoon weights (1.400 and 1.068 gm, respectively) and male cocoon shell ratio (22.708 %) were significantly increased under the effect of antibiotic treatment comparing with control.

Table 1: The mean values of the most important economical silkworm characters.

Parameters	Control	infected	Treated	LSD
Larval Duration (hr)	888 b	1008 a	816 c	21.823
ERR %	95.333 ab	78.667 b	97.333 a	17.823
Larval Weight (gm)	2.331 b	2.114 c	2.631 a	0.188
♀Cocoon weight (gm)	1.266 b	1.239 b	1.400 a	0.124
♀Cocoon Shell weight (gm)	0.230 a	0.205 a	0.252 a	0.054
♀Cocoon Shell %	18.238 a	16.782 a	18.013 a	3.833
♂Cocoon Weight (gm)	1.037 b	1.052 b	1.068 a	0.030
♂Cocoon Shell Weight (gm)	0.204 a	0.211 a	0.232 a	0.054
♂Cocoon Shell %	20.507 b	20.622 b	22.708 a	1.211

In a row, means followed by same letter (s) are not significantly different at 0.01 by LSD

From the obtained results it may be concluded that, Gentamicin increases insect resistance/tolerance and ERR. Also, it improves larval growth which reflects on cocoon weight and silk ratio increment.

The growth- rates of animals have been observed to increase through the feeding of low concentrations of antibiotics. Beneficial effects of antibiotics by modulation of gut micro-flora and influence on mucosal immunity or through altering enzymatic activities has been extensively studied in humans, animals and many insects (Yeung *et al.*, 2002). While in many cases administration of antibiotics was reported to have detrimental effect on intestinal microflora of silkworms which cause adverse effects on the physiological system (Subramanian *et al.*, 2009). So, in the present study it is recommended to apply low concentration of antibiotics. As the antibiotic feed supplementation not only showed prophylactic measures to prevent bacterial infections but also enhanced the feeding nutrition and economical parameters in *Bombyx mori* as suggested by (Sheebha *et al.*, 2008).

The development and economic production of sericulture largely and greatly depends on the metabolic modulations and molecular mechanism of silkworm, besides its genetic composition and immunological resistance (Babu *et al.*, 2005).

Hamamoto *et al.*, (2005) reported that, antibiotics used for clinical purposes may have therapeutic effects on silkworms infected with the pathogens *Staphylococcus aureus* and *Candida albicans*. Kaito *et al.*, (2002) found that when ampicillin (200 µg), oxacillin (200 µg) or vancomycin (200 µg) was injected in the silkworm larvae after injection of the bacterium, at least 90% of the larvae survived for 4 days.

Electrophoresis

Fig. (1) demonstrated the SDS-protein profile of the haemolymph samples of healthy and flacherrie diseased larvae of the investigated groups.

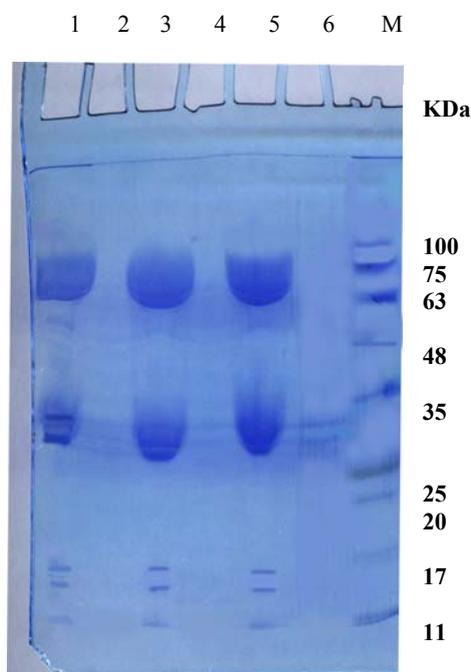


Fig. 1: The electrophoretic pattern of the haemolymph demonstrated the SDS- protein profiles of control, infected and treated samples.

M: molecular weights of marker (KDa)

Lane 1: Healthy sample from control group

Lane 3: Healthy sample from infected group

Lane 5: Healthy sample from treated group

Lane 2 : Flacherrie diseased sample from control group

Lane 4: Flacherrie diseased sample from infected group

Lane 6: Flacherrie diseased sample from treated group

Tables (2 and 3) revealed the computer analysis of protein bands and showed the presence (+) or absence (-) of bands. The electrophoretic pattern of healthy haemolymphal samples from control, infected and treated groups showed a total of 14 protein bands with molecular weights (68.31, 65.08, 54.50, 52.06, 49.08, 45.03, 42.33, 41.43, 31.83, 29.76, 27.76, 14.25, 12.86 and 10.57 KDa, respectively). Whereas, in the flacherrie diseased sample of control group, a total of 8 protein bands were observed with molecular weights (68.31, 65.08, 54.50, 52.06, 45.03, 31.83, 29.76 and 28.98 KDa, respectively). The flacherrie diseased sample of infected group showed 7 protein bands with molecular weights (68.31, 65.08, 54.50, 52.06, 45.03, 31.83 and 29.76 KDa, respectively). Finally, the flacherrie diseased sample of treated group showed 5 protein bands with molecular weights (37.01, 36.12, 31.83, 29.76, 27.76 KDa, respectively).

Table 2: The molecular weight of the different protein bands

MW	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Marker
Band 1	68.31	68.31	68.31	68.31	68.31	37.01	87.75
Band 2	65.08	65.08	65.08	65.08	65.08	36.12	77.66
Band 3	54.50	54.50	54.50	54.50	54.50	31.83	65.45
Band 4	52.06	52.06	52.06	52.06	52.06	29.76	50.02
Band 5	49.08	45.03	49.08	45.03	49.08	27.76	37.31
Band 6	45.03	31.83	45.03	31.83	45.03	-	24.42
Band 7	42.33	29.76	42.33	29.76	42.33	-	21.26
Band 8	41.43	28.98	41.43	-	41.43	-	15.73
Band 9	31.83	-	31.83	-	31.83	-	10.90
Band 10	29.76	-	29.76	-	29.76	-	-
Band 11	27.76	-	27.76	-	27.76	-	-
Band 12	14.25	-	14.25	-	14.25	-	-
Band 13	12.86	-	12.86	-	12.86	-	-
Band 14	10.57	-	10.57	-	10.57	-	-

Table 3: The presence (+), absence (-) of bands and type of bands in all tested haemolymphal samples

MW	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Polymorphism
68.31	+	+	+	+	+	-	polymorphic
65.08	+	+	+	+	+	-	polymorphic
54.50	+	+	+	+	+	-	polymorphic
52.06	+	+	+	+	+	-	polymorphic
49.08	+	-	+	-	+	-	polymorphic
45.03	+	+	+	+	+	-	polymorphic
42.33	+	-	+	-	+	-	polymorphic
41.43	+	-	+	-	+	-	polymorphic
37.01	-	-	-	-	-	+	unique
36.12	-	-	-	-	-	+	unique
31.83	+	+	+	+	+	+	monomorphic
29.76	+	+	+	+	+	+	monomorphic
28.98	-	+	-	-	-	-	unique
27.76	+	-	+	-	+	+	polymorphic
14.25	+	-	+	-	+	-	polymorphic
12.86	+	-	+	-	+	-	polymorphic
10.57	+	-	+	-	+	-	polymorphic

M: molecular weights of marker (KDa)

Lane 1: Healthy sample from control group

Lane 3: Healthy sample from infected group

Lane 5: Healthy sample from treated group

Lane 2 : Flacherrie diseased sample from control group

Lane 4: Flacherrie diseased sample from infected group

Lane 6: Flacherrie diseased sample from treated group

17 bands were recorded in the all tested haemolymphal samples with polymorphism of 88.23%. 2 monomorphic bands were recognized, while the remaining 12 bands were considered as polymorphic ones. 3 bands were recognized as unique bands, as shown in Table (3). The detected polymorphism in the resulted protein profiles could be attributed to some environmental stresses (Sammour *et al.*,

1993) or resulted from the mutational events that alter the performance of their encoding genes as suggested by (Rottenberg *et al.*, 2000). The recorded polymorphism percentage in this study could support this issue.

Unique bands were appeared in the bacterial flacherrie diseased samples for both control and treated groups only. Two unique protein bands with 37.01 and 36.12 KDa were appeared in flacherrie diseased sample of treated group and one unique protein bands with 28.98 KDa in flacherrie diseased sample of control group. The appearance of unique bands in diseased samples may be related to immune proteins.

Bacterial infection can be used to survey the host silkworm genome-wide responses, including its innate immune response to the pathogen at transcriptional level and to provide another detailed comprehension of the interaction between the pathogen and its host. A lot of basal metabolic pathways were significantly modulated. Genes related to poisoning that might be a key to silkworm septicemia, are also regulated (Huang *et al.*, 2009). In response to microbial infection, antimicrobial proteins (AMPs) and lysozymes are rapidly produced primarily in the fat body (FB), and subsequently secreted into the haemolymph to eliminate invading pathogens. In *Bombyx mori*, six different families of (AMPs) have been identified: Cecropin, Attacin, Lebocin, Moricin, Gloverin, and Defensin were up-regulated at 24 hours post the infection (Tanaka and Yamakawa, 2011).

In the present study, there is a common band in all tested samples with molecular weight $29.76 \approx 30$ KDa. This protein type is known as haemolymph protein or larval serum protein (BmLSP) according to Fujiwara and Yamashita (1992), with 262 amino acid residues. Izumi *et al.*, (1981) stated that, a group of structurally related proteins called 30 K proteins, because of their approximate molecular weights of 30 KDa, were found to be stored in the larval haemolymph of silkworms in a stage-dependent fashion. The 30 K proteins are minimally detectable in the haemolymph before day 3 of the fifth larval instar, but become major haemolymph proteins in the early pupal stage because of progressive increase in expression after the 3rd day of fifth instar larvae. Kim *et al.*, (2003) identified the 30 kDa protein as an anti-apoptotic component and inhibit a poptosis in silkworm larvae. It inhibited the virus or chemical-induced apoptosis in human cells as well as insect cells. They recommend that, the recombinant 30 K protein can be effectively used to minimize cell death and consequently increase the productivity by extending the production time of host cells in commercial animal cell culture.

Naletova *et al.*, (1982) identified the protein with 69 KDa as carboxylesterase, which has antigenic activity. In this study, 68.3 KDa protein present in all tested samples except the flacherrie diseased sample from treated group.

Protein with 49.08 KDa may be the paralytic peptide binding protein according to Nakahara *et al.*, (2009) with 421 amino acid residue. This plays an important role in silkworm immunity (Tanaka and Yamakawa, 2011).

Antibiotics used clinically in humans also have therapeutic effects against silkworms injected with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Kaito *et al.*, 2002). Hossain *et al.*, (2006) estimated the bacterial exotoxins that kill silkworms. The 50% lethal dose (LD50) of staphylococcal alpha-toxin is 12 µg/g; and that of staphylococcal beta-toxin is 9 µg/g; that of *Pseudomonas* exotoxin A is 0.14 µg/g; that of diphtheria toxin is 1.1 µg/g.

Most of the LD50 values obtained in silkworm were similar to the reported values in mice, suggesting that silkworms can be used as a model to study the general effects of bacterial exotoxins on multicellular organisms, including humans. In agreement with the opinion of Yamakawa and Tanaka (1999), it is expected that the

study of *B. mori* immunity can contribute in the near future to the medical field developing new antibiotics based on bacterial proteins and to the agricultural field establishing transgenic plants having antimicrobial protein genes.

CONCLUSION

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. Antibiotics that are used in clinic to treat infection in mammals showed therapeutic effect in silkworm larvae infected with bacteria. The silkworm larval infection system may provide a convenient and novel way to discover new antibiotics which have not been found using the existing systems of drug screening and pathogen studies.

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ARABIC SUMMARY

تأثير المضاد الحيوي (جينتاميسين) على مرض الفلاشيرى البكتيري ليرقات دودة القز (بوميكس موراي إل).

سعاد مرسي محمود - رحاب حسني طه - إبراهيم عبد العظيم إبراهيم سعد
قسم بحوث الحرير - معهد وقاية النباتات - مركز البحوث الزراعيه - مصر

هناك العديد من العوامل التي تؤثر في نجاح إنتاج الحرير. ويعتبر مرض الفلاشيرى البكتيري أهمهم على الإطلاق. وأظهرت المضادات الحيوية نتائج جيدة للسيطره على أمراض دودة القز وحصاد محصول متفوق من الشرائق وبالتالي تحسين إنتاج الحرير. هذا البحث يناقش تأثير المضاد الحيوي (جينتاميسين) على مرض الفلاشيرى البكتيري. ويعتبر الجينتاميسين مضاد حيوي رخيص في الأسواق المصريه ويمكن تطبيقه بسهولة من قبل المتدربين و المزارعين .

وقد تبين من خلال دراسته النتائج أن الجينتاميسين يزيد معنويا من معدل كفاءة التربيه (97.333 %) و وزن اليرقه (2.631 جم) و وزن الشرنقه للأنثى (1.400 جم) و وزن الشرنقه للذكر (1.068 جم) وكذلك نسبة المحتوى الحريري للشرنقه الذكر (22.708%).

وقد تم دراسة التباين في أنماط البروتين باستخدام التفريد الكهربى لعينات السائل الدموي لليرقات المختبره. وقد كان هناك تباين كبير بنسبه 88.2% في الطرز البروتينى بين العينات المختبره ، إلا انه تم الحصول على 12 حزمه بروتينيه متعددده الطرز و حزمتين منفرده الطرز في جميع العينات وكذلك ثلاث حزم وحيدده الطرز .