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Effect of mixed bacterial infection on mortality and protein content of *Philosamia ricini* (Donovan) larvae

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KEYWORDS

Silkworm larvae, *Philosamia ricini*, Bacterial infection, Mortality and protein content

A B S T R A C T

In our study day first 5th instar larvae of *Philosamia ricini* (Donovan) multivoltine Titabar Yellow plain pure line eco-race were used for bacterial infection study. Mixed bacterial infection of *Escherichia coli* (NCIM 2137) and *Staphylococcus aureus* (NCIM 2672) were resulted into 25.5% mortality and protein content in hemolymph decreased from 15.6±1.2 mg/ml (healthy larvae) to 9.8±0.64 mg/ml (infected larvae). Biological stress due to mixed bacterial infection resulted in mortality and also changed protein content of hemolymph.

Introduction

Philosamia ricini (Family: Saturniidae) is used for eri silk production as a Vanya silk accounting approximately >70% contribution in total of Vanya silk. India is the largest country producing near about 96% Eri raw silk out of total worlds Eri raw silk, while Assam is producing 65% of Eri raw silk in India (Kumar and Gangwar, 2010). Silkworm larvae are used as model organism in pathogenicity study due to its body size (5 cm), generation time (40-60 days), easy rearing, comfortable handling, low cost rearing, minor space required, outcome of results within a day, high throughput results and non-ethical issue (Evelina *et al.*, 2013). Human pathogenic bacteria cause the disease to the silkworm and will be also cured due to antibiotic treatment similar to mammals. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Vibrio cholerae* cause the diseases to silkworm (Kaito and Sekimizu, 2007). In our research mixture of *Escherichia coli* (Gram negative short rods) and *Staphylococcus aureus* (Gram positive cocci) were used to infect the 5th instar larvae to study the mortality rate and protein content of hemolymph.

Materials and Methods

Preparation of live bacterial antigen

Type culture of *Escherichia coli* (NCIM 2137) and *Staphylococcus aureus* (NCIM 2672) were obtained from the Industrially Important Microorganisms Culture Collection unit of National Chemical Laboratory, Pune. Isolated colonies of *E. coli* and *S. aureus* were used for nutrient broth inoculation and spreading on nutrient agar plate respectively. Overnight broth culture of *E. coli* and colonies of *S. aureus* were used for suspension preparation. Suspensions were centrifuged at 5000 rpm for 10 min and cells were washed using sterile saline. Same process of centrifugation and washing was repeated for two times to remove the metabolites and nutrients. Saline suspensions of these bacteria were used for preparation of 0.5 McFarland standards suspension. Both the suspensions were double time diluted and mixed in 1:1 ratio prior to inject in hemocoel of larvae (Stephen *et al.*, 2005).

Injecting bacterial antigen in hemocoel

Day first 5th instar Eri silkworm larvae *Philosamia ricini* (Donovan) multivoltine Titabar Yellow plain pure line ecological races were used for infection study. Anesthesia was given to larvae by chilling on ice and followed by surface disinfection using 70% ethanol. Larvae were kept on tissue paper for drying. Mixed bacterial suspension (5µl) was injected in the hemocoel of each larva using Hamilton syringe (Yan-Yuan *et al.*, 2011). Total two hundred larvae were injected and reared separately from healthy silkworm larvae, but environmental and nutritional conditions were maintained same as 24°C to 26°C temperature and 80 to 90% relative humidity (Sarkar, 1988 and Sarmah *et al.*, 2012).

Hemolymph sample collection

After 24 hours post infection mortality of healthy and infected larvae were recorded. Live silkworm larvae were used for collection of hemolymph samples (Kyung *et al.*, 2002). Disinfection was done with 70% ethanol before hemolymph collection (Thangamalar *et al.*, 2010). First abdominal leg of larva cut (Hiroko *et al.*, 2007) by using a forceps and free flowing hemolymph were collected into pre-chilled eppendorff tube containing Phenylthiourea crystals (Silva *et al.*, 2010). Immediately these tubes were used for obtaining cell-free hemolymph.

Separation and estimation of proteins

Eppendorff tubes containing hemolymph samples were centrifugation at 200 g for 5min and 20,000 g for 15 min to obtain the cell-free hemolymph (Pawel *et al.*, 2010). Two volumes of 10% TCA in acetone were added to the one volume of cell-free hemolymph in eppendorff tube. Mixture was vortexed on cyclomixture for 10 minutes at cold temperature. Mixture was allowed to stand for 2 hours at -20°C. After incubation sample were centrifuged to 27,000 g for 10 minutes at 4°C. Immediately and carefully supernatant were decanted from microcentrifuge tube. The pellets were dissolved in Ethanol: Ether (1:1 v/v) mixture. Precipitated proteins were dried in air (Smith *et al.*, 1985). Protein precipitate was once again dissolved in 50 µl acidic buffer. Protein concentrations were determination using Micro dilution Bradford method (1976).

Results and Discussion

Mortality rate

Fifty one larvae were died out of 200 initially taken larvae due to mixed bacterial

infection of *E. coli* and *S. aureus*. Infection resulted into 25.5% mortality, whereas non-infected larvae remained healthy (Table 1). In the earlier reports infection by microsporidiosis (Pebrine) caused by *Nosema* species to the tropical tasar silkworm (*Anthereae mylitta*) showed 5% mortality post infection (Velide and Bhagavanula, 2012). Pebrine infected tasar silkworm larvae showed mortality in all instars and also in the rainy, autumn as well as winter season (Mishra *et al.,* 1992). Second and third instar mosquito (*Anopheles stephensi*) larvae were required 31.6 x 10^4 and 7.2 x 10^1 spores of *Beauveria bassiana* as LD50 dose respectively (Geetha and Balaraman, 1999). Infection of *Nosema bombycis* and cross-infection of *N. mylitta* on *Bombyx mori* with respect to temperature and relative humidity showed 36.66 to 57.58 % mortality (Satadal and Buddhadeb, 2008). If infection by viral polyhedral occlusion bodies (OBs) to the larvae of *Bombyx mori* as dose of 1300, 1800 and 2000 OBs/larva resulted in mortality as 17.5, 20 and 23% respectively (Khurad *et al.,* 2005). Infection with AdorGV to the *Adoxophyes orana* larvae *viz.* 1st instar to 5th instar, showed decreased mortality as 100% (1st instar), 96% (2nd instar), 72% (3rd instar), 40 % (4th instar) and 12 % in 5th instar (Karel, 2007). Second instar *Helicoverpa armigera* larvae were treated with *Bacillus thuringiensis* strains recorded mortality in the range of 16.67 to 94.44% (Lalitha *et al.,* 2012). Infection of microorganism has profound effect on mortality. Loss of hunger, dysentery, omitting and dehydration resulted in mortality. Not only infection but other factors are also responsible for mortality.

Protein content

Approximately 0.5 to 0.6 ml free flowing hemolymph was obtained from each larva. In the hemolymph of healthy and infected larvae 15.6±1.2 and 9.8±0.64 mg/ml protein were determined respectively (Table 2). In the past study protein concentration in the hemolymph was decreased in *Achoea janata* L. due to *B. thuringiensis* infection (Govindarajan *et al.,* 1976), while similar infection enhanced protein content of *S. littoralis* larvae (Slama *et al.,* 1983). Microbial infection to *Manduca sexta* larvae changed protein profiles of midgut and hemolymph (Rupp and Spence, 1984). Declined protein content was resulted in the 6th instar larvae of *Peridroma saucia* upon infection by nuclear polyhedroses virus. Fifty percent protein content was decreased in hemolymph of mulberry silkworm larvae due to *Bombyx mori* Infectious Flacherie Virus (BmIFV) infection (Yogananda *et al.,* 2014). Low temperature has induced enhanced protein concentration in Eri silkworm larvae as 17.2 to 25.5 mg/ml while compared with larvae those were reared at normal temperature (Singh *et al.,* 2010). Progress in growth and protein content were directly proportionate, while BmIFV resulted into decreased protein concentration from 6.31 to 22.42 mg/ml (Mamatha and Balavenkatasubbaiah, 2014). Infection of various nematodes has profound effect on decreased protein content of *Spodoptera littoralis* larvae as 17.7 mg/ml of healthy hemolymph and up to 8.13 mg/ml in infected one (Naglaa *et al.,* 2014). In *Plodla interpunctella* larvae infection of *B. thuringinsis* resulted in declined protein content as healthy crude sample 68 mg/ml and infected larvae 58.67 mg/ml (Aboul-Ela *et al.,* 1991).

Very few existing reports were explaining relation of infection with protein content of larvae. Higher protein content is directly proportional to the better metabolic rate. Whole body protein content of Eri silkworm larvae is due to tender castor leaves given as feed.

Table.1 Mortality rate in *Philosamia ricini*

Number of Initial larvae	Number of live larvae after immune challenge
200	149
100%	74.5%
% Mortality = 25.5	

Table.2 Hemolymph protein content of *Philosamia ricini*

Sr. Nos.	Eri silkworm larvae (mg/ml)	
	Healthy	Infected
1	14	9
2	14	9
3	14	9
4	15	9
5	15	10
6	16	10
7	16	10
8	17	10
9	17	11
10	18	11
Mean	15.6	9.8
Mean Deviation	1.2	0.64
Sample variances	2.0444	0.7888
Standard Deviation	1.4298	0.7888
Coefficient of Variation	9.1656	8.0490
Correlation Coefficient	0.006	
Significance Test	0.0171	

Accumulation of biomolecules is mostly dependant on leaves quality, quantity (Ito and Arai, 1963) and rate of consumption by silkworm larvae. Absorption of food constituents' *viz.* proteins and amino acids from digested castor leaves is mostly depending on midgut of larvae. In the castor leaves 18.23 to 28.74 mg/g protein content was observed on the dry weight basis (Manjunath and Sannappa, 2012).

As infected larvae stops feeding, it will resulted into decreased consumption of organic molecules, hence size of larvae remained smaller and appearance of larvae is dull with too much less turgidity. Infection to hemocoel results in infection of midgut as gut is surrounded by hemolymph. In the infected larvae dysentery and vomiting was observed, which concludes that gut was also infected by mixed bacterial infection. Post infection within 24 hours larvae became dull, soft and flaccid before the death. As per the previous reports in the infected larvae lipid and carbohydrate content was found increased, if larvae stops feeding and these two macromolecules are enhanced defiantly protein content should be

declined due to infection as observed in our findings.

Conclusion

In our study biological stress given by mixed infection of *E. coli* and *S. aureus* were resulted into 25.5% mortality, while protein content was also decreased. These findings conclude that mixed bacterial infection should affect the biochemical pathways and became the cause of death of larvae.

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References

Aboul-Ela, R., Kamel, M.Y., Salama, H.S., El-Moursy, A., Abdel-Razek, A. 1991. Changes in the biochemistry of the hemolymph of *Plodia interpunctella* after treatment with *Bacillus thuringiensis*. *J. Islamic Acad. Sci*., 4(1): 29–35.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*., 7(72): 248–254.

Evelina, P., Putri, D.U., Wim, J.Q. 2013. Choosing an appropriate infection model to study quorum sensing inhibition in *Pseudomonas* infections. *Int. J. Mol. Sci.*, 14: 19309–19340.

Geetha, I., Balaraman, K. 1999. Effect of entomopathogenic fungus, *Beauveria bassiana* on larvae of three species of mosquitoes. *Indian J. Exp. Biol*., 11: 1148–1150.

Govindarajan, R.S., Jarayaj, S., Narayanan, K. 1976. Studies on the effect of *Bacillus thuringiensis* Berliner on the castor semilooper, *Achoea janata* L. (Noctuidae, Lepidoptera). *Z ang Ent.*, 80: 191–200.

Hiroko, O., Akiyoshi, M., Kazuhiko, H., Yoshiaki, Y., Isao, M. 2007. Peptidoglycan recognition protein **(**PGRP**)** from eri-silkworm, *Samia cynthia ricini*; protein purification and induction of the gene expression. *Comp. Biochem. Physiol.*, 147(B): 512–519.

Ito, T., Arai, N. 1963. Food values of mulberry leaves for the silkworm *Bombyx mori* L. determined by means of artificial diets. I. Relationship between kinds of mulberry leaves and larval growth. *Bull. Seric. Exp. Stn. Japan*, 18(4): 226–229.

Kaito, C., Sekimizu, K. 2007. A silkworm model of pathogenic bacterial infection. *Drug Discov. Ther.*, 1: 89–93.

Karel, P. 2007. Influence of Baculovirus AdorGV on the mortality of larvae and pupae of summer fruit tortrix *Adoxophyes orana* in laboratory conditions. *Plant Protect. Sci.*, 43(3): 94–102.

Khurad, A.M., Mahulikar, A., Rathod, M.K., Rai, M.M. 2005. Infection of nucleopolyhedrovirus in the larval rudiments of gonads of silkworm, *Bombyx mori* L. *Indian J. Seric.*, 44: 159–164.

Kumar, R., Gangwar, S.K. 2010. Impact of varietal feeding on *Samia ricini* Donovan in spring and autumn season

of Uttar Pradesh. *ARPN J. Agricut. Biol. Sci.,* 5(3): 46–51.

Kyung H.Y., Kyu N.K., Joon H.L., Heui S.L., Sang H.K., Kyung Y.C., Myung H.N., In, H.L. 2002. Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, *Galleria mellonella*, *Bombyx mori*, *Agrius convolvuli*. *Dev. Comp. Immunol.,* 26: 707–713.

Lalitha, C., Muralikrishna, T., Sravani, S., Devaki, K. 2012. Laboratory evaluation of native *Bacillus thuringiensis* isolates against second and third instar *Helicoverpa armigera* (Hubner) larvae. *J. Biopest,* 5(1): 4–9.

Mamatha, M., Balavenkatasubbaiah, M. 2014. Biochemical changes during the progressive infection of bmifv in the silkworm, *Bombyx mori* L. *International J. Plant, Anim. Environ. Sci.,* 4(2): 372–378.

Manjunath, K.G., Sannappa, B. 2012. Evaluation of castor ecotypes of selected regions of the Western Ghats of Karnataka, India through Bio-Chemical Assay. *Int. J. Sci. Res.,* 3(358): 2045–2051.

Mishra, C.S.K., Nayak, B.K., Dash, M.C. 1992. Larval mortality of Indian Tasar silkworm, *Antheraea mylitta*, Saturnidae due to pebrine infection. *J. Lepidoptera Soc.,* 46: 106–109.

Naglaa, F.A., Amna, M.H., Maklad., Samia A.Y., Shaker, M.A. 2014. Biochemical effects of *Steinernema feltiae*, *Steinernema riobrave* and *Heterorhabditis bacteriophora* on *Spodoptera littoralis* larvae. *Egypt. Acad. J. Biolog. Sci.,* 6(1): 23–34.

Pawel, M., Agnieszka, Z., Malgorzata, C. 2010. A different repertoire of *Galleria mellonella* antimicrobial peptides in larvae challenged with bacteria and fungi. *Dev. Comp. Immunol.,* 34: 1129–1136.

Rupp, R.A., Spence, K.D. 1984. Protein alteration in *Mandusa sexta* midgut and hemolymph following treatment with a subiethal dose of *Bacillus thuringiensis* crystal endotoxin. *Insect Biochem.,* 15: 147–154.

Sarkar, D.C. 1988. Eri culture in India. *CSB Publ. Bangalore*, 28.

Sarmah, M.C., Ahmed, S.A., Sarkar, B.N., Debaraj, Y., Singh, L.S. 2012. Seasonal variation in the commercial and economic characters of Eri Silkworm, *Samia ricini* (Donovan). *Munis Entomol. Zool.,* 7(2): 1268–1271.

Satadal, C., Buddhadeb, M. 2008. Influence of temperature and humidity on infection of *Nosema bombycis* and cross-infection of *Nosema mylitta* Chakrabarti and Manna, 2006 in growth and development of mulberry silkworms, *Bombyx mori* L. *Int. J. Ind. Entomol*., 17(2): 173–180.

Silva, J.L.C., Barbosa, J.F., Bravo, J.P., de Souza, E.M., Huergo, L.F., Pedrosa, F.O., Esteves, E., Daffre, S., Fernandez, M.A. 2010. Induction of a gloverin-like antimicrobial polypeptide in the sugarcane borer *Diatraea saccharalis* challenged by septic injury. *Braz. J. Med. Biol. Res.,* 43: 431–436.

Singh, A., Sharma, R.K., Sharma, B. 2010. Low temperature induced alterations in certain biochemical constituents of 5th instar larvae of *Philosamia ricini* (Lepidoptera: Satunidae). *Open Access Insect Physiol*., 2: 11–16.

Slama, H.S., Sharaby, A., Ragei, M. 1983. Chemical changes in the hemolymph of *Spodoptera littoralis* (Lep: Noctuidae) as affected by *Bacillus thuringiensis*. *Entomophaga,* 28: 331–337.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem*., 150(1): 76–85.

Stephen, J.C., Ronald, J.H., Yvette, S.M., Jose, H.O., Ivonne, D.R., Robert, L.S., Susan, E.S., Carol, A.S. 2005. Manual of antimicrobial susceptibility testing. American Society for Microbiology.

Thangamalar, A., Subramanian, S., Muthuswami, M., Mahalingam, C.A. 2010. Fate of bacteria and on set of immune response in silkworm, *Bombyx mori* L. *J. Biopesticides.* 3(1): 47–50.

Velide, L., Bhagavanulu, M.V.K. 2012. Studies on susceptibility to microsporidiasis in the Ecoraces of Tasar Silkworm, *Anthereae Mylitta* drury with reference to phenotypic and cocoon characters. *Asian J. Exp. Biol. Sci.*, 3(3): 493–497.

Yan-Yuan, B., Jian, X., Wen-Juan, W., Ying, W., Zu-Yao, L., Chuan-Xi, Z. 2011. An immune-induced Reeler protein is involved in the *Bombyx mori* melanization cascade. *Insect Biochem. Mol. Biol.*, 41: 696–706.

Yogananda, V.N.M., Bharathi, R., Jayaram, G.N., Lokesh, G. 2014. Critical biochemical analysis in different body tissues in three commercial silkworm (*Bombyx mori* L.) Races. *Asian J. Natural Appl. Sci.*, 3(2): 20–30.