Vol 4 Issue 11 May 2015

ISSN No :2231-5063

International Multidisciplinary Research Journal

Golden Research Thoughts

Welcome to GRT

RNI MAHMUL/2011/38595 **ISSN No.2231-5063**

Golden Research Thoughts Journal is a multidisciplinary research journal, published monthly in English, Hindi & Marathi Language. All research papers submitted to the journal will be double - blind peer reviewed referred by members of the editorial board.Readers will include investigator in universities, research institutes government and industry with research interest in the general subjects.

International Advisory Board

Editorial Board

Address:-Ashok Yakkaldevi 258/34, Raviwar Peth, Solapur - 413 005 Maharashtra, India
Cell : 9595 359 435, Ph No: 02172372010 Email: ayisrj@yahoo.in Website: www.aygrt.isrj.org

International Recognized Double-Blind Peer Reviewed Multidisciplinary Research Journal
Golden Research Thoughts
ISSN 2231-5063 Impact Factor :3.4052(UIF)
Volume - 4 | Issue - 11 | May - 2015 Available online at www.aygrt.isrj.org

EFFECT OF MIXED BACTERIAL INFECTION ON MORTALITY AND PROTEIN CONTENT OF SILKWORM LARVAE (*BOMBYX MORI* L.)

Bajare Jitendra Shahaji
Assistant Professor, Department of Biotechnology, V. G. Shivdare College of Arts, Commerce and Science, Solapur.

Short Profile

Bajare Jitendra Shahaji is working as an Assistant Professor at Department of Biotechnology in V. G. Shivdare College of Arts, Commerce and Science, Solapur. He has completed M.Sc. Microbiology. He has Professional Experience 12 years.

Co - Author Details :

Dulange Sanjay Mallikarjun[2] and Thorat Prakash Ramrao[3]
[2]Assistant Professor, Department of Microbiology, Adarsh Mahavidyalaya, Umarga.
[3]P. G. Department of Microbiology and Research Centre, Shri Shivaji Mahavidyalaya, Barshi.

ABSTRACT:

In our study day first 5th instar larvae of *Bombyx mori* (L.) mutivoltine race Kolar Gold (PM X CSR2) cross breed were used for bacterial infection study. Mixed bacterial infection of *Escherichia coli* (NCIM 2137) and *Staphylococcus aureus* (NCIM 2672) were resulted into 28% mortality and protein content in hemolymph decreased from 39.3±1.56 mg/ml (healthy larvae) to 26.8±1.04 mg/ml (infected larvae). Biological stress due to mixed bacterial infection resulted in mortality and changed protein content of hemolymph.

KEYWORDS

Silkworm larvae, Bombyx mori, bacterial infection, mortality and protein content.

1.INTRODUCTION :

Bombyx mori L. (Family: Bombycidae) are predominantly used for production of 90% of worldwide silk (Krishnaswami, 1990). 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) was 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 Mulberry silkworm larvae *Bombyx mori* (L.) mutivoltine race Kolar Gold (PM X CSR2) cross breed 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 27°C temperature and 80 to 90% relative humidity (Krishnaswami, 1990).

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 a*l., 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 six larvae were died out of 200 initially taken larvae due to mixed bacterial infection of *E. coli* and *S. aureus.* Infection resulted into 28% mortality, whereas non-infected larvae remained healthy (Table 1). As per the earlier report increased mortality was found in outdoor reared larvae compared to indoor ones. Microbial infections, rain and shifting of larvae were responsible for mortality (Kumar and Shamitha, 2013). Microsporidiosis infection caused 20 to 28% mortality in tropical tasar silkworm (Velide and Rao, 2011). Infection of 1 x 105 conidia/ml *Beauveria bassiana* showed 5 to 90% mortality in mulberry silkworm (Chandrasekharan and Nataraju, 2011). Individual infection of (Bombyx mori Infectious Flacherie Virus) BmNPV and *S. faecalis / S aureus* showed ≈ 9 to 56% mortality in *Bombyx mori* (Selvakumar and Datta, 2013). Infection of microorganism has profound effect on mortality. Loss of hunger, dysentery, omitting and dehydration resulted in mortality.

Protein content:

Approximately 0.3 to 0.4 ml free flowing hemolymph was obtained from each larva. In the hemolymph of healthy and infected larvae 39.3±1.56 and 26.8±1.04 mg/ml protein was determined respectively (Table 2). In the past study of infection by BmIFV hemolymph protein were decreased by 50 % within 12 days over the control hemolymph protein. Three Bombyx mori races were showed variation in hemolymph protein concentration as 34.23, 38.20 and 40.12 mg/ml (Yogananda *et al*., 2014). As growth progresses there was increase in protein concentration from 6.31 to 44.11 mg/ml within 12 days, while infection with BmIFV resulted into decreased protein concentration from 6.31 to 22.42 mg/ml (Mamatha and Balavenkatasubbaiah, 2014).

Infection study using entomopathogenic nematode to the *Spodoptera littoralis* larvae also affected the protein concentration viz. healthy larvae had 17.47 mg/ml protein in the hemolymph, while post infection protein concentration were decreased in *Steinernema feltiae* infection (12.1

mg/ml), *Steinernema riobrave* (9.95 mg/ml) infection and *Heterorhabditis bacteriophora* infection as 8.13 mg/ml (Naglaa *et al*., 2014). Infection by *B. thuringinsis* to *Plodla interpunctella* larvae also showed influence on the hemolymph protein concentration. Untreated hemolymph of said larvae as control crude sample and control TCA precipitated sample were showing 68 mg/ml and 50.4 mg/ml protein concentration respectively, while post infection hemolymph protein were decreased as treated crude sample (58.67 mg/ml) and treated TCA precipitated sample contained 44.8 mg/ml (Aboul-Ela *et al.*, 1991).

Higher molecular weight polypeptides has rise to synthesis of complex proteins. It plays a vital role in the formation of structures and to do various biological functions. When carbohydrates and lipids are not available (adverse condition) protein will also utilized as energy source. Modification or modulation of proteins during stress condition induces alternative pathways in the tissues (Assem and Hanke, 1983). Very few references are available explaining the relation of infection and its effect on protein metabolism. Presence of high protein content is the indication of better metabolic activity in the tissue. Higher protein content in the whole body parts of silkworm is because of feeding of tender leaves instead of medium or coarse leaves (Hisao, 1994). Accumulation of biomolecules is mostly dependant on leaves quality and quantity consumed by silkworm (Ito and Arai, 1963). Absorption of food constituents' viz. proteins and amino acids from digested mulberry is mostly depending on midgut of larvae (Seo *et al.*, 1985). Diseased larvae stopped the feeding i.e. consumption of organic molecules decreased, hence dull and smaller sized larvae were observed in the infected larvae. Infection in the hemocoel also affect the midgut because larvae have open circularity system i.e. hemolymph is direct surrounds the midgut, it will results into infection to gut. Infected larvae showed dysentery and vomiting of gut juice, hence diseased larvae became dull, soft and flaccid previous to death. In the general observation lipid content and carbohydrate content mostly increased during the infection. If carbohydrate and lipid content increases protein should be declined, similar results were observed in our study.

CONCLUSION:

In our study biological stress given by mixed infection of *E. coli* and *S. aureus* were resulted into 28% mortality, while protein content was also decreased. These findings conclude that biological, physical, environmental and chemical stress to the larvae should affect the biochemical pathways and death of larvae.

ACKNOWLEDGEMENT:

Authors are thankful to Mr. M. B. Patil, Former acting Principal, V. G. Shivdare college of Arts, Commerce and Science and Dr. Madhukar Fartade, Princile, Shri Shivaji Mahavidyalaya, Barshi for providing laboratory facility for this study.

Compliance with Ethical standards

Ethical approval: In this article used biological material silkworm larvae has no ethical issue (Evelina et al., 2013).

Conflict of interest: All authors have no conflict of interest for publication.

REFERENCES:

1.Aboul-Ela R., Kamel M.Y., Salama H.S., El-Moursy A. and Abdel-Razek A. (1991). Changes in the biochemistry of the hemolymph of Plodia interpunctella after treatment with Bacillus thuringiensis. Journal of Islamic Academy of Sciences. 4(1):29-35.
2.Assem H. and Hanke W. (1983). The significance of the aminoacid during osmotic adjustment in teleost fish- I. Changes in the Euryhaline Sarotherodon mossambicus. Comp. Biochem. Physiol. 74(A):531-536.
3.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.
4.Chandrasekharan K. and Nataraju B. (2011). Beauveria bassiana (Hyphomycetes: Moniliales) Infection during Ecdysis of Silkworm Bombyx mori (Lepidoptera: Bombycidae). Mun. Ent. Zool. 6(1):312-316.
5.Evelina P., Putri D.U. and Wim J.Q. (2013). Choosing an Appropriate Infection Model to Study Quorum Sensing Inhibition in Pseudomonas Infections. Int. J. Mol. Sci. 14:19309-19340.
6.Hiroko O., Akiyoshi M., Kazuhiko H., Yoshiaki Y. and Isao M. (2007). Peptidoglycan recognition protein (PGRP) from eri-silkworm, Samia cynthia ricini; protein purification and induction of the gene expression. Comparative Biochemistry and Physiology. 147(B):512-519.
7.Hisao A. (1994). Principles of Sericulture. CRC Press. 1-266.
8.Ito T. and 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.
9.Kaito C. and Sekimizu K. (2007). A silkworm model of pathogenic bacterial infection. Drug Discov. Ther. 1:89-93.
10.Krishnaswami S. (1990). Improved Method of Rearing Young Age (Chawki) Silkworms, Central Sericulture Research and Training Institute, Mysore. Reprint of Bulletin. 3.
11.Kumar S.G. and Shamitha G. (2013). Studies on Larval mortality: Diseases, Pest and Predator menace in Outdoor and Indoor reared Tasar Silkworm, Antheraea mylitta Drury (Daba TV). Res. J. Animal, Veterinary and Fishery Sci. 1(4):1-7.
12.Kyung H.Y., Kyu N.K., Joon H.L., Heui S.L., Sang H.K., Kyung Y.C., Myung H.N. and In H.L. (2002). Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, Galleria mellonella, Bombyx mori, Agrius convolvuli. Developmental and Comparative Immunology. 26:707-713.
13.Mamatha M. and Balavenkatasubbaiah M. (2014). Biochemical changes during the progressive infection of bmifv in the silkworm, Bombyx mori L. International Journal of Plant, Animal and Environmental Sciences. 4(2):372-378.
14.Naglaa F.A., Amna M.H.Maklad., Samia A.Y. and 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.
15.Pawel M., Agnieszka Z. and Malgorzata C. (2010). A different repertoire of Galleria mellonella antimicrobial peptides in larvae challenged with bacteria and fungi. Developmental and Comparative

Immunology. 34:1129-1136.
16.Selvakumar T. and Datta R.K. (2013). Role of Bacteria (Streptococcus faecalis & Staphylococcus aureus), Viruses and Their Combined Infection In Causation of Flacherie under Different Environmental Conditions. Sericologia. 53(1):54-62.
17.Seo R.W., Youn C.Y., Kang C.S. and Kim H.R. (1985). A Study on protein pattern of haemolymph during last larval and pupal stages of Bombyx mori L. Bull. Entomol. Res. 11:153-164.
18.Silva J.L.C., Barbosa J.F., Bravo J.P., de Souza E.M., Huergo L.F., Pedrosa F.O., Esteves E., Daffre S. and Fernandez M.A. (2010). Induction of a gloverin-like antimicrobial polypeptide in the sugarcane borer Diatraea saccharalis challenged by septic injury. Brazilian Journal of Medical and Biological Research. 43:431-436.
19.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. and Klenk D.C. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem. 150(1):76-85.
20.Stephen J.C., Ronald J.H., Yvette S.M., Jose H.O., Ivonne D.R., Robert L.S., Susan E.S. and Carol A.S. (2005) Manual of antimicrobial susceptibility testing. American Society for Microbiology.
21.Thangamalar A., Subramanian S., Muthuswami M. and Mahalingam C.A. (2010). Fate of bacteria and on set of immune response in silkworm, Bombyx mori L. Journal of Biopesticides. 3(1):47-50.
22.Velide L. and Rao P.A. (2011). Studies on the impact of microsporidiosis on tropical tasar silkworm Anthereae mylitta Drury. J. Appl. Biosci. 44:2994-2999.
23.Yogananda V.N.M., Bharathi R., Jayaram G.N. and Lokesh G. (2014). Critical Biochemical Analysis in Different Body Tissues in Three Commercial Silkworm (Bombyx mori L.) Races. Asian Journal of Natural & Applied Sciences. 3(2):20-30.

TABLES:

Table 1: Mortality rate in *Bombyx mori*

Number of Initial larvae	Number of live larvae after immune challenge
200	144
100%	72%
% Mortality = 28	

Table 2: Hemolymph protein content of *Bombyx mori*

Sr. Nos.	Mulberry silkworm larvae (mg/ml)	
	Healthy	Infected
1	37	25
2	38	25
3	39	26
4	40	26
5	41	27
6	42	27
7	37	27
8	38	28
9	39	28
10	42	29
Mean	39.3	26.8
Mean Deviation	1.56	1.04
Sample variances	7.1333	3.4666
Standard Deviation	2.6708	1.8618
Coefficient of Variation	6.7960	6.9473
Correlation Coefficient	0.0698	
Significance Test	0.1981	

Publish Research Article
International Level Multidisciplinary Research Journal
For All Subjects

Dear Sir/Mam,

We invite unpublished Research Paper,Summary of Research Project,Theses,Books and Book Review for publication,you will be pleased to know that our journals are

Associated and Indexed,India

- International Scientific Journal Consortium
- OPEN J-GATE

Associated and Indexed,USA

- EBSCO
- Index Copernicus
- Publication Index
- Academic Journal Database
- Contemporary Research Index
- Academic Paper Databse
- Digital Journals Database
- Current Index to Scholarly Journals
- Elite Scientific Journal Archive
- Directory Of Academic Resources
- Scholar Journal Index
- Recent Science Index
- Scientific Resources Database
- Directory Of Research Journal Indexing

Golden Research Thoughts
258/34 Raviwar Peth Solapur-413005,Maharashtra
Contact-9595359435
E-Mail-ayisrj@yahoo.in/ayisrj2011@gmail.com
Website : www.aygrt.isrj.org