Fate of bacteria and on set of immune response in silkworm, *Bombyx mori* L.

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ABSTRACT

Dynamics of immune responses in five cross breeds of mulberry silkworm, *Bombyx mori* was assessed by following through pattern of bacterial colonization *in vivo*. The colony forming units (cfu/ml) were measuring at specified time intervals at 0h, 6h, 12h, 24h, 30h, 36h and 48h after inoculations of non-pathogenic bacteria, *Escherichia coli* (Kanamycin resistant) @ 10^6 cfu/ml into larvae of silkworm breeds during third, fourth and fifth stages. The colonization pattern shows that there was a surge in colony counts at 6h post immunization followed by a sharp decline. At 48h post immunization, bulk of the ingested bacteria was cleared off in most of the breeds. Comparison of the pattern of *E. coli* colonization in different breeds during third, fourth and fifth instars revealed that the rate of clearance of ingested bacteria was in the order of fifth instar > fourth instar > third instars. Among the breeds APM2 x APS12 and APM3 x APS12 recorded faster clearance of bacteria as depicted by 40-50% reduction in colony forming units at around 12– 24 h. Significant differences in clearance of ingested marker bacteria *E. coli* shows that some of the cross breeds like APM2 x APS12 and APM3 x APS12 are more immune to invasions of bacteria than popular cross breed like PM x CSR2.

Key words: Silkworm *Bombyx mori* L*., Escherichia coli,* bacterial colonization

INTRODUCTION

Diseases are a major yield limiting factors in silkworm crop. The most important character that determines the commercial success of any silkworm breed is its tolerance to diseases under adverse conditions. Tolerance or susceptibility to diseases are characterized by inherent immune responses of the breed. Immune response activities of haemolymph are responsible for protecting the insects from pathogenic and non pathogenic bacteria. Swiftness of clearance of ingested bacteria is determined by the combined action of cellular and humoral defenses of insects. Immune responses of insects can be studied by following the pattern of colonization of bacteria in the haemolymph *in vivo*. Studies with *Hylophora cecropia* showed that the injected bacteria, *Escherichia coli* could be cleared at around 48 h after post injection (Faye *et al*., 1975; Flyg *et al*., 1987; Kaaya *et al*., 1987). Induction kinetics of antibacterial activity reveal that after the initial lag period, immune system is activated which the bacterial multiplication in check keeps leading to clearance of inoculated bacteria (Faye *et al*., 1978). Colonization of bacteria followed similar pattern in several species of insects including, *Helicoverpa zea* (Cheung *et al*., 1978); *Manduca sexta* (Dunn and Drake, 1982); *Drosophila* (Flyg *et al.,* 1987); *Glossina* sp. (Kaaya *et al.,* 1987); *H.armigera* (Subramanian, 1994). Immune dynamics of indigenous

silkworm breeds was ascertained in the present study by following the colonization pattern of bacteria with a known drug resistant marker.

MATERIALS AND METHODS
Silkworm

The different Indian silkworm breeds *viz.,* PM x CSR2, APM1 x APS8, APM 2 x MVT, APM2 x APS12 and APM3 x APS12 were collected from the Government Grainage Centre, Coimbatore, Andhra Pradesh State Sericulture Research and Development Institute, Hindpur. The given races were used for experiment. Silkworm breeds were incubated under laboratory conditions of temperature 26±3° C and RH 70-80% and allowed to hatch. After hatching, the larvae were reared in rearing trays and fed on V1 variety of mulberry leaves. By following standard rearing technologies, rearing of the silkworm breeds was done (Dandin *et al*., 2000).

Bacteria, media and immunization

Required glass wares were sterilized in a hot air oven at 180°C for one hour. Required growth medium and broth were sterilized in an autoclave at 15 lb pressure for 20 minutes. Isolation, purification, inoculation and other microbial works carried out in laminar air flow chamber under aseptic condition. To track the pattern of

colonization of bacterial *E. coli* strain with a drug resistant marker (Kanamycin resistance) was used in the studies. Nutrient agar medium and nutrient broth were used for culturing *E. coli* K^R. Freshly collected mulberry leaves were dipped in 50 ppm *E. coli* K^R broth culture filtrate (10^6 cfu / ml). The treated leaves were shade dried and offered during first feeding after moults during third, fourth and fifth instar.

Collection of haemolymph

The larvae were swabbed with 70 per cent ethyl alcohol. Tip of the first proleg was snipped off, using sterlized blade / scissors. Required amount of haemolymph was collected in sterilized eppendorf tubes. A pinch of phenylthiourea (PTU) was added (to avoid melanization) in the haemolymph samples and stored at -20° C until further analysis.

Pattern of colonization of bacteria in silkworm

Third, fourth and fifth instar larvae of silkworm breeds ingested with *E. coli* K^R were bled at definite time intervals and haemolymph samples were plated on nutrient agar plates. Bacterial titre (cfu) was estimated after incubation at 37^oC for 24 h.

RESULTS

Pattern of bacterial colonization was followed through in different silkworm breeds during growth stages by measuring the colony forming units at specified time intervals in third, fourth and fifth instar. Table 1 represents the pattern of *E. coli* colonization in different silkworm breeds at third instar. General perusal of the data shows that there was a gradual decline in colony forming units (cfu) over time irrespective of the breeds as revealed by the negative correlation coefficient values for the parameters of bacterial counts over time. Temporal dynamics of bacterial colonization followed a complex pattern as illustrated by divergent R values for different breeds. Comparatively faster clearance in bacteria is noticed in the silkworm cross breed APM2 X APS12 with more than 50 % reduction in cfu at 30h compared to base level of 10^6cfu/ml.

Colonization pattern of *E. coli* in different silkworm breeds during fourth instar show significant negative correlation between bacterial colony counts over time (Table 2). Initially there was a surge in colony counts at 6 h post in all the breeds tested. Significant reduction (about 50 per cent reduction over base level count) in bacterial colonization was noticed at about 30h post inoculation in all the breeds noticed. At 48 h post immunization bulk of the ingested bacteria was cleared off in most of the breeds as revealed by the cfu of 1.40×10^2 (APM2 x APS12) to 2.92×10^2 (PM x CSR2).

Table 3 depicts the pattern of *E. coli* colonization in different silkworm breeds. There was a strong negative correlation between bacterial colony counts and time. Initial colonization pattern showed an upward trend at 6 h post immunization. There was nearly 100-fold reduction in cfu at 12 h post inoculation. About 50 per cent reduction in bacterial colony counts was noticed at 24h post inoculation in the silkworm cross breed, APM2 x APS12. At 48 h post inoculation, bulk of the bacteria was cleared from the haemolymph in APM2 x APS12 and APM3 x APS12. Comparing the pattern of *E. coli* colonization in different breeds during third, fourth and fifth instars it was observed that the rate of clearance of ingested bacteria was in the order of fifth instar > fourth instar > third instar. Comparative analysis of the cfu/ml over time indicates that among the breeds APM2 x APS12 and APM3 x APS12, recorded faster clearing of bacteria as depicted by 40-50 % reduction in cfu at around 24 h post inoculation.

DISCUSSION

The efficiency of coordinated immune response could be gauged by the *in vivo* colonization of invading bacteria. Colonization of bacteria in silkworm breeds *in vivo* was

Table 1. *E. coli* colonization in different silkworm crossbreeds during third instar (cfu / ml)

Time interval (h)	*E. coli* (K^R) colony counts (cfu / ml)				
	PM x CSR2	APM1 x APS8	APM2 x MVT	APM2 x APS12	APM3 x APS12
0	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6
6	2.8×10^6	2.7×10^6	2.8×10^6	2.4×10^6	2.6×10^6
12	2.1×10^5	2.4×10^6	2.7×10^5	2.0×10^5	2.1×10^5
24	3.5×10^4	4.9×10^5	4.9×10^4	4.3×10^4	4.5×10^4
30	2.9×10^4	4.4×10^4	4.6×10^4	3.9×10^3	4.2×10^3
36	3.2×10^3	3.7×10^3	3.3×10^3	3.3×10^2	3.5×10^2
48	1.5×10^3	2.9×10^2	2.9×10^2	1.0×10^2	1.1×10^2
R value	- 0.4369	- 0.3961	- 0.3970	- 0.4321	- 0.4274

Table 2. *E. coli* colonization in different silkworm crossbreeds during fourth instar (cfu / ml)

Time interval (h)	*E. coli* (K^R) colony counts (cfu / ml)				
	PM x CSR2	APM1 x APS8	APM2 x MVT	APM2 x APS12	APM3 x APS12
0	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6
6	1.8×10^6	2.1×10^6	2.3×10^6	1.7×10^6	1.9×10^6
12	1.9×10^5	2.1×10^5	2.4×10^5	1.9×10^5	1.9×10^5
24	3.9×10^4	4.5×10^4	4.9×10^4	3.2×10^4	3.6×10^4
30	2.9×10^3	3.6×10^3	3.8×10^3	3.3×10^3	3.4×10^3
36	1.9×10^3	2.8×10^3	2.9×10^3	2.4×10^3	2.2×10^3
48	2.9×10^2	2.1×10^2	2.3×10^2	1.4×10^2	1.6×10^2
R value	-0.5813	-0.5597	-0.55322	-0.5692	-0.5754

probed through an *E. coli* strain marked with kanamycin resistant gene. Temporal dynamics of *E. coli* colonization was worked out by drawing haemolymph at specified time interval. Since the ingested bacteria carries a molecular marker, by plating the colonies on selective media with kanamycin *in vivo* concentration could be tracked.

The extent of clearance of inoculated bacteria varies with the breeds. The breeds APM2 x APS12 and APM3 x APS12 registered comparatively faster clearance of ingested bacteria as revealed by 40-50% reduction in cfu at around 24 h post inoculation. Among the larval instars the rate of clearing of ingested *E. coli* was in the order of fifth instar > fourth instar > third instar. Temporal dynamics of bacterial colonization followed a static pattern during third instar competed to that of fourth and fifth instars as revealed by the R values.

Studies with *H. cecropia* on the fate of injected *E. coli* showed that there was a lag period o7f 6-8 h followed by rapid clearing of bacteria (Faye, 1978). Reduction in concentration of circulating bacteria was noticed at varying time intervals following ingestions of *E. coli* D 31 into *M. sexta* (Dunn and Drake, 1983). *In vivo* experiments in several insects proved that clearing of bacteria occurred at 24 h post inoculation (Gagen and Ratcliffe, 1976; Subramanian, 1994). Fate of ingested bacteria into the insect haemocoel showed that the insects

have acquired an 'immune' state following exposure to viable non-pathogenic bacteria (Boman *et al.,* 1974). During the initial time lag of 6-12 h cellular responses phagocytose or nodulate the bacteria and subsequently newly synthesized antibacterial proteins lyse the invading bacteria. The pioneering studies from Boman (1995), unraveled the physiological mechanisms of humoral defense in insects involving *de novo* synthesis of antibacterial proteins. Faster clearance of inoculated bacteria at about 24 h probably indicates the onset of humoral immune response triggering the synthesis of antibacterial proteins. Humoral responses in *B. mori* have well been studied with 35 antimicrobial peptide genes have so far been detected from this species. An assemblage of antibacterial proteins including Moricins, Lebocins, Enbocins, Cecropins, Attacins and Defencins constitute humoral response of *B. mori* taking cure of ingested bacteria (Cheng *et al.,* 2006). Moreover *E. coli* can be used to expresse Dihydrolipoamide dehydrogenase (DLDH) activity in *B. mori* (Huo *et al.,* 2010). Dihydrolipoamide dehydrogenase (DLDH), a flavin-dependent oxidoreductase is essential for energy metabolism in insects. Hence expression of *E. coli* in cross breeds of silkworm is a desirable feature.

Multivoltine Indian silkworm breeds are generally considered to be sturdy and tolerant to diseases probably

Table 3. *E. coli* colonization in different silkworm crossbreeds during fifth instar (cfu / ml).

Time interval (h)	*E. coli* (K^R) colony counts (cfu / ml)				
	PM x CSR2	APM1 x APS8	APM2 x MVT	APM2 x APS12	APM3 x APS12
0	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6
6	1.6×10^6	1.6×10^6	1.7×10^6	1.6×10^6	1.6×10^6
12	1.9×10^5	1.5×10^5	1.6×10^5	1.2×10^4	1.3×10^4
24	2.5×10^4	2.9×10^4	2.9×10^4	2.0×10^3	2.1×10^6
30	1.5×10^4	1.8×10^3	1.9×10^3	1.6×10^3	1.7×10^3
36	1.9×10^3	2.4×10^2	2.4×10^2	1.9×10^2	2.1×10^2
48	1.2×10^2	1.4×10^1	1.5×10^1	1.3×10^4	1.3×10^4
R value	-0.9843	-0.9827	-0.9819	-0.9694	-0.9610

owing to their strong immune responses to disease causing pathogens. Multivoltine cross breeds occupy a major share of commercial sericulture. As field stability of these cross breeds are determined by their tolerance to diseases under varied climatic conditions, it is pertinent to ascertain their immune dynamics by assessing immune response parameters. Clearance of bacteria *in vivo* was taken as a parameter to compare the breeds in the present study. Comparative analysis of bacterial colonization in different silkworm breeds in the present study showed that some of the breeds *viz.,* APM2 x APS12 and APM3 x APS12 recorded faster clearance of ingested bacteria as depicted by 40-50 % reduction in cfu at 24 h post inoculation. Faster clearance of ingested bacteria indicates the swiftness of immune response in these breeds. Further studies are required to measure other immune response parameters like changes in differential haemocyte counts, Prophenol oxidase activity, Cecropin and Lysozyme units to ascertain the immune dynamics of these breeds.

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