Bacteriology of Flacherie in *Bombyx mori* L

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ABSTRACT:

Sericulture practice in Tamilnadu is affected by flacherie attack on the mulberry silk worm, Bombyx mori. Bacteria causing flacherie infection were isolated from disease developed silkworm, *Bombyx mori*. Antibiotic sensitivity studies were conducted on the bacterial isolates. In the present study 43 species of bacterial strains were isolated from different organs in flacherie affected silkworms. The total heterotrophic bacterial count was found high in the midgut region, where digestive microbes predominate. The haemolymph and silkglands were found to be the major centres for the high multiplication of bacteria. The antibiogram for the isolates , *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Proteus mirabilis and Enterobacter cloacae* revealed that these organisms had developed resistance to a many antibiotics particularly to Gentamycin.

Key words: ; *Bombyx mori*; Flacherie; sericulture ; antibiogram; antibiotic sensitivity

INTRODUCTION:

The silkworm, *Bombyx mori*, is exposed to number of disease causing microorganisms at different levels, causing the development of disease and mortality. These diseases have been grouped under four major categories, *viz*., microsporidian, viral, bacterial and fungal. Pasteur(1870) reported bacteria as an etiological agent for silkworm flacherie. Pasteur ascribed the pestilential epizootics among silkworms to t5wo symptomatically different diseases,pebrine and flcherie. Cuboni and Garibini (1890) reported *Bacillus cubonianus* as the causative agent of flacherie. Metalnikov and Chlorine (1928) found *Serratia marcescens* to induce one type of flacherie. In China Hartman (1931) reported that the bacterium, *Bacillus bombysepticus* cause one type of flacherie. Steinhaus (1949) observed *Bacillus mycoides* and *Bacillus laterosporus* as pathogenic agents for silkworm.Hukuhara (2014) reported that Flacherie is one of the great scourges of sericulture.Karthikairaj et.al., (2013) reported the prevalence of bacterial flacherie in some parts of Tamilnadu and suggested plant remedies for that

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Further a number of bacteria were reported as flacherie causing agents (Selvakumar,2013;Balavenkadasubbiah et al.,2015;Anusha and Bhasker 2016 and Bebitha et al., 2016)). As

flacherie outbreak is one of the main reason for the loss in sericulture practices in Tamil nadu, an attempt has been made to find out the microbial consortium responsible for this disease and to trace out the antibiotic sensitivity for the isolates.

MATERIALS AND METHODS:

The silkworm hybrid of Pure Mysore X NB4 D_2 (Multivoltine X Bivoltine) of *Bombyx mori* was selected for the present study.

Isolation, identification and enumeration of bacterial pathogens in bacterial flacherie infected larvae.

The bacterial flacherie infected larvae (III- V instar) were collected from the sericulture farms in Tirunelveli district. The infected worms were surface sterilized by treating serially with the following disinfectant solutions, as per (Cantwell 1974).

1. 70 – 95% Ethyl alcohol for 2 minutes.
2. 5.25% Sodium hypochlorite for 3 – 5 minutes.
3. 10% Sodium thiosulphate for 3 – 5 minutes.
4. Three changes of sterile distilled water.

The surface-sterilized silkworms were dissected out aseptically and the gut, silk gland and haemolymph were collected carefully in sterile eppendorff tubes. The fore-gut, mid-gut and hind-gut were isolated separately. From the gut and the silk gland about 1 gram tissue was taken out separately and was homogenized aseptically. Then the homogenates were made to 100 ml using 1% sterile peptone water. Further serial dilutions were made using 9ml of the same dilution. 1ml of an aliquot of the homogenate was streaked on nutrient agar plates. The streaked plates were incubated for one day at a temperature of 36 ± 2^{o} C and relative humidity of 60 ± 5%. The growth of bacterial colonies was observed and an individual colony was isolated. Representatives of morphologically dissimilar well-isolated colonies were selected at random from the nutrient agar plates with samples of gut, silk gland and haemocoel and sub cultured to check the purity after noting morphology and pigmentation of the colony. Then the pure bacterial isolates were again sub cultured in nutrient agar slants. The slants were stored at 4 ^{o} C in a refrigerator and periodical sub culturing was done to maintain the viability of the bacterial strains. The bacterial cultures were also identified using API identification procedure of Biomerieux, England (a fully automated system for bacterial identification) for confirming the manual method of identification.

For enumerating the bacteria load in the haemolymph, the collected haemoloymph, was serially diluted with 1% sterile peptone water and incubated for 72 hours. The number of bacterial colonies formed was counted using bacteriological colony counter. The plates which contain 30 – 300 colonies were selected for enumeration of total plate count. The Bacterial populations were expressed as number of Colony Forming Units (CFU) per gram of sample analysed.

Antibiotic sensitivity study was carried out for 5 species of bacterial pathogens isolated from silkworm larvae that are resistant to preliminary screening and also on the basis of their abundance in different organs. It includes *Escherichia coli* from skin, *Serratia marcescens* – mid – gut, *Staphyllococcus aureus*- haemocoel; *Proteus mirabilis* – silkgland and *Enterobacter cloacae* – hind - gut region. Using 24 hour old cultures, the sensitivity of these bacteria to different antibiotic drugs were determined using API automation unit (Biomerieux, UK) of bacterial identification and sensitivity testing.

RESULTS:

In the present study 43 types of bacterial strains were isolated from different organs of the silkworms (Table 1) Of the different organs tested a high bacterial diversity was observed in mid gut (22 species of bacterial), the major centre for digestive activities. The bacterial diversity is less in foregut (6) and hind gut (4). Of the 43 species of bacteria isolated, the bacteria *Serratia marcescens* was found in all organs except skin. Next to *S.marcescens*, *Klebsiella ornitholytica* was found in more organs (3 organs). About 9 species of bacteria are seen in two organs and the rests are present in only one organ.

The mean total heterotrophic bacterial count in foregut, mid gut, hind gut, silk gland and haemocoel in the normal and bacterial flacherie infected worms are presented in (Table 2). Of the five tissues analysed the total heterotrophic bacterial count was high in mid gut region, where digestive microbes predominates. When compared to the tissues of the control worms, the total heterotrophic bacterial counts in disease developed worms were higher. Of the five tissues the percentage change in haemolymph was very high (302.7%). Next to haemolymph the bacterial pathogens were high in the silk gland (200%).

The results of *in vitro* screening of antibiotics against *S.marcescens, E.coli, S.aureus, E.cloacae* and *P.mirabilis* after 24 hours exposure. (Table 3) revealed that all these organisms had developed resistance to variety of antibiotics particularly to Pencillin. Except *E.coli* ,all the other bacterial species showed resistance to Gentamycin. The bacteria S.marcescens showed resistance to many antibiotics. For some of the antibiotics Fosfomycin, Netilmicin, Ticar , Clav.AC., chloramphenicol, Gentamicin and Nitrofurantoin, the bacteria *E.coli* isolated from the integument was highly sensitive but *S.marcescens* showed resistance to these antibiotics. Similarly the drug Nitrofurantoin was sensitive to the organism *S.aureus* but resistant to *S.marcescens.* The sensitive and resistant drugs details are given in the (Table 3).

DISCUSSION:

When compared to the previous works, Chitra *et al.,* (1975) identified 36 species of bacteria in different organs. In the present study 43 species of bacteria were identified. Such a report on high diversity of bacterial strains helps well in developing suitable chemical treatments for silkworm diseases.

Balavenkatasubbiah et al.,(2015)reported that there is an urgent need for disease management in sericulture to overcome the loss the Indian farmers are facing. Selvakumar,(2013) reported that adverse environmental conditions such as temperature and humidity are considered important predisposing factors for flacherie and cocoon crop loss.

Bacteria infect the silkworm larvae mostly through the mouth and digestive tract and less commonly through the egg, integument and tracheae (Govindan and Devaiah ,1995). They may also enter by means of parasites and predators, careless management, overcrowded trays, accumulation of faces in the trays, rough manipulation during the transport (Lu Yup and Liu Fu– 1991and Vootla etal., 2013). In the alimentary canal the bacteria produce enzymes that damage the mid-gut epithelium and enable the bacteria to enter the haemocoel. Most of the insect pathogenic bacteria occur in the family's Bacillaceae, Pseudomonadacea, Enterobacteriaceae, Streptococcaceae and Micrococcaceae (Tanada and Kaya 1993). Members of the family Bacillaceae produce endoscopes and most of the insect pathogen of this family includes the genera *Bacillus* and *Clostridium.*

Serratia marcescens is a facultative pathogen (Bell *et al.*, 1981) remains beneficial inside the gut and not pathogenic when present in digestive tract in small numbers (Sikorowski 1985). The gut p^H and environment inhibits its pathogenicity. If there is any damage to the gut wall this parasite *S.marcescens* invades the haemocoel and cause many health problems. *S.marcescens*, a red-pigmented microorganism with high motility was observed in the guts of the normal worm and in the gut and haemocoel of diseased worms.

Also the presence of many human pathogens present in the infected larvae further invites a serious attention. The human pathogens present in silkworm larvae may cause illness in Sericulturists as an occupational illness.

The present study alerts the sericulture farmers to adopt proper bed cleaning, hygienic methods of handling the larvae, sterilization of utensils and rearing room, personal hygiene of the workers, providing clean disease and pest free mulberry leaves, proper ventilation, non utilization of sewage or dirty water for mulberry cultivation etc.

Thus the exogenous factors in the farmer's sericulture field had altered the endogenous disease resistance properties in the digestive juice, haemolymph, granular cells of blood and cuticular lipids etc. and the silkworms were highly susceptible to bacterial flacherie.

Table 1 Table showing meaning ± SD total heterotrophic bacterial count in different Organs of control and bacterial flacherie infected worms.

Organs	Control worm cfu/gm	Diseased worm cfu/gm	Percentage change from control
Fore- gut	$10.4 \times 10^5 \pm 3.1$	$20.3 \times 10^5 \pm 2.4$	95
Mid-gut	$30.6 \times 10^7 \pm 4.3$	$48.4 \times 10^7 \pm 0.1$	58
Hind -gut	$28.0 \times 10^7 \pm 2.4$	$36.3 \times 10^7 \pm 2.9$	29
Silk gland	$2.1 \times 10^4 \pm 0.7$	$6.3 \times 10^4 \pm 1.3$	200
Haemocoel	$4.3 \times 10^4 \pm 4.3$	$18.4 \times 10^4 \pm 2.8$	302.7

Table 2 Bacterial strains isolated from different regions of flacherie infected worms

Sl. No.	Organisms	Presence of bacteria in different tissues						
		Fore-gut	Mid-gut	Hind-gut	Silk gland	Haemo-lymph	Skin	Bacteria and No. of tissues
1	*Staphylococcus aureus*	+	+	-	-	-	-	2
2	*S. epidermis*	+	-	-	-	-	-	1
3	*S. albus*	-	-	-	+	-	-	1
4	*Bacilus thuringiensis*	+	+	-	-	-	-	2
5	*B.bombycis*	+	+	-	-	-	-	2
6	*B. noctuarum*	-	+	-	+	-	-	2
7	*B. orpheus*	-	+	-	-	-	-	1
8	*B. cereus*	+	-	-	-	-	-	1
9	*Streptococcus faecium*	-	-	+	-	-	-	1
10	*Streptococcus faecalis*	-	-	+	-	-	-	1
11	*Achromobacter delmarvae*	-	-	-	-	+	-	1
12	*A. Superficialis*	-	+	-	-	+	-	2
13	*A.cloacae*	-	+	-	-	-	-	1
14	*Aerobacter aerogenes*	-	-	-	-	+	-	1
15	*Aerobacter hydrophia*	-	-	-	+	-	-	1
16	*Acinetobacter*	-	-	-	+	-	+	2
17	*Alcaligenes xyloxidans*	-	+	-	-	-	-	1
18	*Enterobacter cloacae*	-	-	-	+	-	-	1
19	*Enterobacter aerogenes*	-	+	-	-	-	-	1
20	*E. coli*	-	-	-	-	-	+	1
21	*E.freundi*	-	-	-	-	+	-	1
22	*Proteus inconstana*	-	+	-	-	-	-	1
23	*P.morgani*	-	+	-	-	-	-	1
24	*P.vulgaris*	-	+	-	-	-	-	1
25	*P.mirabilis*	-	+	-	-	-	+	2

26	*Pseudomonas boreopolis*	-	+	-	-	-	-	1
27	*P.ovalis*	-	+	-	-	-	-	1
28	*P.aeroginosa*	-	-	-	-	+	+	2
29	*Kilebsiella ornitholytica*	-	+	-	+	-	+	3
30	*K.pneumoniae*	-	-	-	-	+	+	2
31	*Yersinia pseudotuberculosis*	-	-	-	-	-	+	1
32	*Y.enterocolitica*	-	-	-	-	-	+	1
33	*Serratia liquefaciens*	-	+	-	-	-	-	1
34	*S.marcescens*	+	+	+	+	+	-	5
35	*S.piscotorum*	-	-	-	-	+	+	2
36	*Lactobacillus plantarum*	-	-	-	+	-	-	1
37	*L.cellobiosus*	-	-	-	+	-	-	1
38	*Micrococcus alimentans*	-	-	-	-	-	-	0
39	*M.candidus*	-	+	-	-	-	-	1
40	*M.flavus*	-	+	-	-	-	-	1
41	*M.freenden*	-	+	-	-	-	-	1
42	*M.roseus*	-	+	-	-	-	-	1
43	*M.vanans*	-	-	+	-	-	-	1
No.of isolates		6	22	4	8	8	9	

Table 3 Antibiogram of selected microbes isolated from IV instar larvae of silkworm infected with bacterial flacherie

ANTIBIOGRAM	ORGANISMS TESTED				
	Escherichia coli	*Staphylococcus aureus*	*Serratia marcescens*	*Enterobacter cloacae*	*Proteus mirabills*
Highly sensitive	Imipenem Piper Tazobactum Amikacin Netilmicin Chloramphenicol Nitrofurantonin Isepamycine Cefoxitin Ticar/ Clav. AC Tobramycin Gentamyin Fosfomycin	Erythromycin Clindamycin Tet/Cyclines Vancomycin Minocycline Teicoplanin, Rifampicin Nitrofurantoin Dalfopristin/Quinupur Fusidic Acid	Imipenem Piper Tazobactum Amikacin Isepamycine Cefoxitin, Fosfomycin	Imipenem Amikacin Fusidic Acid Vancomycine Netilmicin Cefoxitin	Tet/Cyclines Isepamycine Minocycline PiperTazobactum Tobramycin Clindamycin
Moderately sensitive	Nil	Nil	Nil	Nil	Nil
	Ticarcillin Cotrimoxazole Oflaxacin Amo/Pencil, GR.A Colistin, Cefepime	Cotrimoxazole Penicillin Coag- Oxacillin Oxacillin Levofloxacin	Ticarcillin, Fosfomycin Cotrimoxazole, Ticar/Clav. Ac., Oflaxacin,	Fosfomycin Penicillin Colistin Cefexime Aztreonum	Cotrimoxazole Colistin Pencillin Ticarcilin Oflaxacin

Resistant	Cefepirome Cefuroxime Cefexime Ciprofloxacin Ceftazidime Aztreonam	Nor /Quinolones 2G Gentamyicin	Amo/Pencill.GR.A, Netilmicin, Chloramphenicol Colistin, Cefepime, Cefepirome, Tobramycin, Gentamicin, Nitrofurantoin, Cefuroxime, Cefexime, Ciprofloxacin.	Netilmicin Oxacillin Gentamycin	Coag-oxacillin Gentamycin

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