Studies on silkworm diseases—III epizootiology of a septicemic disease of silkworms caused by *Serratia marcescens*

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Received on December 8, 1977 ; Revised on January 16, 1978

Abstract

Serratia marcescens can cause a lethal septicemia even when it enters the silkworm through the oral route if an associative organism identified as *Staphylococcus* sp. is present in the gut. If a few larvae infected with *Serratia marcescens* are released amongst healthy larvae that have been fed with the associative *Staphylococcus*, mortality due to *Serratia* is seen to manifest. These studies have given certain interesting clues to the occurrence of bacterial epizootics in the silkworms.

Key words : *Serratia marcescens*, Silkworm diseases, Septicemia, Epizootiology, Microbial interactions.

1. Introduction

The silkworm (*Bombyx mori* L.), the most important beneficial insect of Karnataka State, is afflicted by several diseases, many of which are endemic. Periodically one disease or other occurs on an epizootic scale. The factors facilitating such incidences are not always known[1]. Among them, a disease, one which occasionally attains serious proportions, is "Kenchu". The dead larvae in certain cases develop brownish specks and turn 'red' typical of *Serratia* infections. Soon after death, the internal organs remain still intact.

From dead larvae collected from sericultural farms a number of strains of *Serratia* were isolated by us.

Serratia as a pathogen on silkworms and many other invertebrates is known for a long time (it has been reported on silkworms in 1796). In fact, the organism has its own credentials as a potential non-sporulating bacterial insecticide[2,3]. Yet it is widely believed, that while the organism is a serious pathogen in the laboratory or insectary reared larvae, natural epizootics caused by it rarely occur. Even in the laboratory/insectary sudden epizootic outbreaks have been attributed more to the development of the pathogen already present in the larvae (due to certain rearing conditions) than due to the organism spreading from individual to individual[4].

Another interesting feature of *Serratia* infections is that the disease is a septicemia and is very lethal when injected directly into the blood but is often harmless when introduced orally.

What then causes the outbreaks that are observed in the farms ? In our country, not much seems to have been done except identifying *Serratia marcescens* as the causative agent of a similar disease popularly known as " Rangi " in the West Bengal area. What predisposes the larvae then, to *Serratia* infections ? Different approaches were made to answer the question, and our work was nearly over when we found that some of our results had been anticipated[5]. These aspects will be discussed later.

2. Materials and methods

Silkworms

The silkworms of different races were reared on mulberry leaves under normal conditions. The layings (eggs) were obtained from Government grainages. Occasionally older larvae were obtained from Government or private farms and used in the study.

Isolation of microorganisms

Affected larvae were collected from different farms and the bacteria were isolated from blood as well as the midgut following the usual insect pathological techniques[6]. All the organisms were purified and characterised following the standard microbiological methods[7, 8]. The required strains were used in the study and the cultures were maintained on nutrient agar.

Testing for pathogenicity

The organisms were grown in nutrient broth at 30° C. Eighteen to 24 hr old cultures were used. The optical density was determined in a spectronic 20 Bausch and Lomb colorimeter at 600 nm and the viable cell count by plating out serial dilutions.

(*a*) *Injection*: From the cultures, cell suspensions in physiological saline of required cell density were prepared. With a microsyringe, the required volume (10, 25, 50 µl) was injected (24 gauge needle) into the haemocoel in the third or fourth abdominal segment and the wound was immediately sealed with collodion.

(*b*) *Feeding*: Suspensions of required cell concentrations were prepared and 0·1 to 0·5 ml was uniformly spread on mulberry leaves. The moisture was allowed to dry and the required number of larvae were allowed to feed on the infected leaves.

The controls were fed or injected with similar amounts of saline, medium or distilled water as the case may be.

All the larvae received mulberry leaves *ad libitum*. The onset of the disease and mortality were observed at regular intervals and the surviving larvae were allowed to spin cocoons and emerge.

The details regarding the age of larvae, the silkworm race and the bacterial strain used, etc., are detailed in the respective tables. Generally larvae were used on the second day after the IV moult. The data were analysed as per recommended methods[6].

Associative effect of Serratia *and other organisms*

Since *Serratia* was found to be poorly lethal to silkworms by the oral route, attempts were made to see whether the presence of certain other organisms predisposes the larvae to succumb to *Serratia*. For this purpose, organisms that were isolated from '*sappe*' diseased larvae[9] as well as those isolated during the study were checked.

These were cultivated in nutrient broth as detailed earlier and required numbers of *Serratia* and the test organisms were mixed, applied on mulberry leaves and fed to the larvae.

The effect of time and order of administering the organisms on the manifestation of the disease was also studied. For this purpose, the larvae were fed with either *Serratia* or the test organisms and one or two days later, the other organism was fed, and the course of the disease was followed.

Spread of Serratia

Whether a *Serratia* infection can spread in a colony was tested by leaving a few larvae artificially infected with *Serratia* amidst a population of uninjected larvae and following the outbreak of the disease.

Fifty or hundred silkworm larvae of the required age were taken and these were left untreated or fed with a test organism used in the previous experiment (Main batch). A separate set of larvae from the same stock was taken and these were infected with *Serratia* either by injection or by feeding. These larvae were clearly marked with paint and these were left amidst the " Main batch " larvae prepared as above. Different ratios of unmarked " Main batch " larvae and the marked *Serratia* treated larvae were tried—*i.e.*, 50/5 ; 50/10 ; 100/5, 100/10.

The marked larvae were kept at random and no attempt was made to control the position or the movement of the larvae. All the larvae were then fed with normal mulberry leaves. The onset of disease among the unmarked population was then regularly observed, and mortality due to *Serratia* septicemia was scored.

3. Results and discussion

Isolotion and characterization

From the dead larvae with reddish tinge several chromogenic and non-chromogenic bacteria were isolated. Both chromogenic and non-pigmented forms of *Serratia marcescens* could be recovered from the blood and the midgut of the larvae. Bacterial strains not belonging to *Serratia* were also found to occur and these were likewise

purified and studied in the usual way[7, 8] and were identified as belonging to *Staphylococcus* sp. and *Bacillus* sp. (Table I).

Table I

Bacteria isolated from silkworms died of "Kenchu"

Organisms	Blood	midgut
Red pigmented *Serratia*	7	3
Non-pigmented *Serratia*	4	4
Staphylococcus sp.	4	4
Bacillus sp.	1	...

a – number of different strains, obtained from pinkish dead larvae.

Pathogenicity

Several strains were obtained and a few strains only were taken for detailed study and the strains used are denoted at the appropriate places. *Serratia* was found to be poorly toxic to silkworms when introduced through the oral route at any of the larval stages (Table II). The few dead larvae did not show the characteristic symptoms of

Table II

Effect of age on oral toxicity of Serratia *to silkworms*†

Instar	Number gone to the next instar				
	Inoculation number of cells per larva 10^6	4th	5th	Number spinning	Emergence of moth
3	10	49	46	45	40
	1	50	48	38	32
	0	50	50	50	47
4	10	...	50	47	43
	1	...	50	47	32
	0	...	50	47	42
5	10	...	...	50	45*
	1	...	...	50	49
	0	...	...	50	50

† Fifty larvae of Mysore × C'nichi were fed with *Serratia* strain VK I cells spread on mulberry leaves

* Three dead larvae showed typical "Kenchu" symptoms.

Serratia infections, except in one instance. Unlike this, the age of the worms plays an important role in the susceptibility of the larvae to enteric pathogens[10].

The dead larvae showing the symptoms of *Serratia* were dissected and the organisms present in the blood and the gut were isolated and purified.

Though it is well known that *Serratia* is poorly toxic to insects through the oral route, the resistance shown by *B. mori* is far higher than what has been observed in grasshoppers, codling moth,[11] etc. On the other hand, the Gypsy moth *Lymantria dispar* also required several million viable cells to cause mortality through feeding[12, 13]. In contrast, all the different races of the silkworms were highly susceptible when *Serratia* was introduced by injection into the blood. The LD_{50} was found to be 1·2 cells/larva which is lower than what has been reported in certain other insects[11, 13, 14]. We also found there was no difference in the virulence of the pigmented and non-pigmented strains of *Serratia*. The different races of silkworms checked were also equally susceptible (Table III). Similar observations have been made in the case of the cockroach[14], the boll weevil[15], the desert locust[16] and a hymonopterous parasite[17].

Table III

Effect of feeding or injecting Serratia *cells on different races of silkworms**

Race	Number of survivors+	
	Fed	Injected
Nanung	50	0
C'nichi	49	0
Kalimpong	...	0
Mysore × C'nichi	47	0
Mysore× KA	50	0

* Fifty V instar larvae were used in the feeding and twenty in the injection experiments. In all cases controls showed no mortality. 10^6 cells were fed or 10^4 cells were injected per larva. Separate controls were kept for each route of infection fed or injected with sterile saline. Strain used VK1.

+ After five days. The *Serratia* injected larvae were dead in 24 hr.

The pathogenicity of organisms earlier reported by Chitra *et al.* as enteric pathogens were tested for their virulence by microinjection into the hemocoel and were found to be ineffective by that route. Heat killed cells of *S. marcescens* VK1, and its culture filtrates revealed no toxic effects (Table IV).

Table IV

Effect of injecting the silkworms with different bacteria

Particulars	Number of cells	Survivors
Aerobacter cloacae	10^4	10
Pseudomonas ovalis	10^4	10
Serratia VK1	10^4	0
Heat-killed VK1	10^4	10
VK1-culture filtrates*	10 μl	10
Pigment extract†	5 μl	10
Medium	10 μl	10
H_2O	10 μl	10
Ethanol	5 μl	10

Ten larvae in the fifth instar were used in each experiment. Two races of silkworms NN6D, Kalimpong were used and the results were identical.

* The culture supernatant from above was used.

† Ten ml of a 24 hr old culture was centrifuged and the cells were extracted with 5 ml of ethanol and 5 μl were injected into the larvae.

These results then left the periodic large scale outbreak of *S. marcescens* infections unexplained. We considered the possibility whether the uzi fly which is a parasite on silkworms can act as a vector and inject the pathogen directly into the hemocoel of the host. Fortunately uzi fly, a serious menace to sericulture in certain other sericultural tracts, does not occur in the Karnataka area. Neither did we observe the fly maggots at any time within the larvae. It is interesting to mention here that Bell *et al.* reported that a braconid parasite could transmit *Serratia* cells from one host to another[16].

Steinhaus has stressed that often it is the rearing conditions facilitating the development of the pathogen already present in the organism that are responsible for the manifestation of a disease, than a pathogen gaining entry afresh[4]. *Serratia* has been called only a potential pathogen by Bucher[3].

At this stage we started investigating whether any of the enteric pathogens, earlier described by Chitra *et al.*, could aid in the entry of *Serratia* into the blood from the gut.

Associative effect of Serratia *and* Staphylococcus

Studies showed that none of the "*sappe*" isolates when fed along with *S. marcescens* VK1 exerted any associative effect. Then we turned our attention to some of the non-*Serratia* isolates that were obtained from larvae died of *Serratia* infections. While we were at it, we came across an interesting paper by Kodama and Nakasuji[5]. They found that a strain of *Streptococcus faecalis-S. faecium* when fed along with *Serratia piscatorum* facilitated the latter to invade the hemocoel. *Serratia piscatorum* by itself was non-pathogenic by the oral route, but was highly virulent when injected.

We continued our studies and found that two isolates VK42 and VK45, identified as belonging to *Staphylococcus* sp. were potential synergists of *Serratia*. *Serratia marcescens* VK1 together with VK42 or VK45 was highly lethal, whereas, singly none of these strains caused any damage (Table V). The dead larvae showed typical symptoms. The order of feeding was found to be important. *Serratia* is invasive only when the associative organism is already present in the gut. If, instead, *Serratia* is fed first to be followed by *Staphylococcus*, no damage is caused.

Table V

*Effect of non-*Serratia *organisms on the manifestation of septicemia due to* S. marcescens *introduced through feeding**

	NIL (control)	*S. marcescens* VK1		
		VK1 and test organism fed together	VK1 fed 2 days prior to the test organism	VK1 fed 2 days after feeding the test organism
NIL (control)	39, 46	40 (0)	42	50
VK42—				
Staphylococcus sp.	33	26 (16)	31 (0)	26 (20)
Pseudomonas ovalis	37	34 (0)	32 (0)	24 (0)
Escherichia fruendii	31	27 (0)	...	25 (0)
VK45—				
Staphylococcus sp.	37 (0)	18 (10)	32 (2)	18 (32)

Mysore × C'niche or Mysore × KA races of silkworms in the V instar (50) were used. Feedings (10^6 cells/larva) were given on the 1st and 3rd day of the fifth instar. In case of double infections on two separate days the first organism was fed on the first day and the other organism on the third day. Controls were given uninoculated medium.

* Number surviving. Figures in paranthesis denote number dead with *Serratia* symptoms. Studies with different test organisms were conducted on different days and the data have been pooled.

Steinhaus had observed that mixed infections were more effective than pure infections[19]. Stephens also found it to be so in grasshoppers[11]. Curiously in his studies with mixed infections with *P. aeruginosa* and *S. marcescens*, he found that in the insects died of septicemia only, either one of the species predominated in the blood. It is likely the associative *Staphylococcus* damages the midgut membranes, so that *Serratia* can penetrate into the blood.

Spread of Serratia *in colony*

Additional studies were conducted to investigate the mode of spread of *Serratia* infections through a colony. For this purpose, a few larvae injected with *Serratia* were left amongst healthy larvae which have been variously treated. The results showed (Table VI) that *Serratia* could spread through a colony if the larvae harboured associative organisms. In other words *Serratia* infections can occur in an epizootic form if an associative organism is present in the gut.

Table VI

Spread of Serratia *infections through a colony*

Marked larvae treatment*	Number of larvae spinning cocoons			
	Nil	VK1 fed	VK1 injected	Fed with VK1 and VK42
Unmarked main batch treatment**				
Nil	49	36	43	50
VK42 (Fed)	38	40	29†	28†
Mixed suspensions "Sappe" isolates (Fed)	46	49††	32†	48

* Five larvae were treated as stated, marked with paint and kept in trays containing 50 larvae.

** 50 larvae/treatment.

† All dead larvae showed typical symptoms of *Serratia* infections.

Crowding facilitates faster spread, for in the treatments where 100 larvae were kept, mortality was greater than in treatments where only 50 larvae in trays of similar dimensions. Over-crowding has been recognised as an important stress factor in the manifestation of diseases in insect larvae, particularly in the silkworms[20]. Depending upon the mode of infection of marked larvae, we observed an interesting difference in the rate of spread of the disease amongst healthy larvae. The marked larvae injected with

S. marcescens (which results in their death) there is greater mortality among the 'healthy' larvae than the introduced larvae fed with *Serratia*. This might be because *Serratia* multiplies fast in the blood and high populations are released into the environment after death by these larvae.

4. Conclusions

Though *Serratia* infection occurs only sporadically; at times it attains serious proportions. Both the associative *Staphylococcus* and the *Serratia* have been isolated from mulberry leaves in our laboratory (unpublished data). The establishment of the disease will then be decided by the population of these organisms and the relative time of their ingestion.

5. Acknowledgements

Parts of these studies were supported by grants from the Central Silk Board, Government of India, Bombay. We are also thankful to Professor B. R. Seshachar, Sri. P. Venugopalan Nair, former Director of Sericulture, Government of Karnataka and other officials of the Department for their help and co-operation. We are thankful to Dr. C. Chitra for going through the manuscript.

References

1. CHITRA, C., KARANTH, N. G. K. AND VASANTHARAJAN, V. N. — Diseases of the mulberry silkworm, *Eombyx mori* L. *J. Sci. Ind. Res.*, 1975, 34, 386–401.

2. ANGUS, T. A. — Symposium on microbial insecticides: 1. Bacterial pathogens of insects as microbial insecticides. *Bact. Rev.*, 1965, 29, 364–372.

3. BUCHER G. E. — Potential bacterial pathogens of insects and their characteristics. *J. Insect Pathol.*, 1960, 2, 172–195.

4. STEINHAUS, E. A. — *Serratia marcescens* Bizio, as an insect pathogen. *Hilgardia*, 1959, 28, 351–380.

5. KODAMA, R. AND NAKASUJI, Y. — Further studies on the pathogenic mechanism of bacterial diseases in gnotobiotic silkworm larvae. *Res. Commun. Inst. Fermentation, Osaka*, 1971, 5, 1–9.

6. MARTIGONI, M. E, AND STEINHAUS, E. A. — *Labaratory Exercises in Insect Microbiology and Insect Pathology.* 1961, Burgess Publishing Co. Minnepolis.

7. Society of American Bacteriologists — *Manual of Microbiological Methods*, 1957, McGraw-Hill, New York.

8. BREED, R. S., MURRAY, E. G. D. AND SMITH, N. R. — *Bergey's Manual of Determinative Bacteriology*, 7th edn., 1957, Williams and Wilkins Co., Baltimore.

9. CHITRA, C., ARUNA, B., KARANTH, N. G. K. AND VASANTHARAJAN, V. N. — Studies on "Sappe" disease of the silkworm *Bombyx mori* L.–I. *Curr. Sci.*, 1973, **42**, 373–376.

10. CHITRA, C., KARANTH, N, G. K. AND VASANTHARAJAN, V. N. — Studies on "Sappe" disease of the silkworm *Bombyx mori* L.–II. *J. Invert. Pathol.*, 1974, **24**, 248–252.

11. STEPHENS, J., M. — Note on the effect of feeding grasshoppers with two pathogenic species of bacteria simultaneously. *Can. J. Microbiol.*, 1959, 5, 313–316.

12. PODGWAITE, J. D. AND COSENZA, B. J. — A strain of *Serratia marcescens* pathogenic for larvae of *Lymantria dispar* : Characterisation. *J. Invert. Pathol.*, 1976, **27**, 185–190.

13. PODGWAITE, J. D. AND COSENZA, B. J. — A strain of *Serratia marcescens* pathogenic for larvae of *Lymantria dispar*: Infectivity and mechanisms of pathogenicity. *J. Invert. Pathol.* 1976, **27**, 199–208.

14. HEIMPEL, A. M. AND WEST, A. S. — Notes on the pathogenicity of *Serratia marcescens* Bizio for the cockroach *Blatella germanica* L. *Can. J. Zool.*, 1959, 37, 169–172.

15. SLATTEN, B. H. AND LARSON, A. D. — Mechanisms of pathogenicity of *Serratia marcescens* : I. Virulence for adult Bollweevil. *J. Invert. Pathol.*, 1967, **9**, 78–81.

16. STEVENSON, J. P. — Epizootiolgy of a disease of the desert locust *Schistocerca gregaria* caused by non-chromogenic strains of *Serratia marcescens* Bizio. *J. Invert. Pathol.*, 1959, **1**, 232–244.

17. BRACKEN, G. K. AND BUCHER, G. E. — Mortality of Hymonopterous parasite caused by *Serratia marcescens*. *J. Invert. Pathol.*, 1967, **9**, 130–132.

18. BELL, J. V., KING, E. G. AND HAMALLE, R. J. — Interactions between Bollworms, a braconid parasite and the bacterium *Serratia marcescens*. *Ann. Ent. Soc. Amer.*, 1974, 67, 712–714.

19. STEINHAUS, E. A. (Ed.) — *Insect Pathology*, 1963, **1**, Academic Press, New York.

20. STEINHAUS, E. A. — Crowding as a possible stress factor in insect disease. *Ecology*, 1958, **39**, 503.