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RESEARCH ARTICLE

Isolation and Characterization of Serratia species in Silk Rearing Environment

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Abstract

Different isolates obtained from the study area (Chickkabalpura(13° 26' N and 77° 46' E), Ramanagara (12° 54' N and 78° 02' E) and Kolar(13° 09' N and78° 11' E) Districts of Karnataka) in seasons of rain, winter, and summer were examined for *Serratia Sp*., septicemia pathogen of silkworm. These samples were subjected to microbiological isolation on minimal media and presumptive identification was done by visualizing pigment production in culture plate. Rhizosphere soil isolates obtained in rainy season produced red pigment, so compared with other known red pigmented and non-pigmented septicemia causing bacteria by injecting into the haemocoel of Silkworm larvae for identification of septicemia symptoms. Soil isolate with septicemia symptoms tested for Koch postulates. Soil isolate showed similarities with other septicemia bacteria in symptoms as well as in the colony and biochemical characteristics.Results of soil isolate obtained compared with specimen species data of the Bergy's Manual of Determinative Bacteriology (9th Edition) and found that soil isolate to be *Serratia plymuthica*. Our study will augment the knowledge of bacterium which later helps in prevention and cure of septicemic disease of silkworm. This bacterium can also be used in the fermentation process, production of prodiogisin and production of different enzymes.

Introduction

Serratia species are ubiquitous in nature found in water, soil, plants and animals. Serratia associated with plants release substances having following effects like growth promoting, antifungal, pathogenic and helps soil bacterium to fix nitrogen symbiotically in associating with plants (Ashelford *et al.*, 2002; Jeong *et al.*, 2010). Serratia used as industrial strain so applied in fermentation process for the production of various enzymes (Fu *et al.*, 2004; Khardenavis *et al.*, 2009). *Serratia marcescens* is also an opportunistic pathogen of immune compromised humans causing diseases (Richards *et al.*, 2000). In insects diagnosed both in pathogenic and non pathogenic form but most of pathogenic Serratia species were found to produce red colored substance prodiogisin in an insects and wild environment. *Serratia* strains isolated from insects often produce more proteolytic enzymes than do wild isolates from other sources (Decedue *et al.*, 1979; Miyata *et al.*, 1981).

In 1963 Bucher stated gram-negative, 0.5-1.0µm, rod-shaped, non-sporing, motile bacterium with peritrichous flagella, and produces prodiogisin pink to red colored non-diffusible pigment in its natural environment. *Serratia marcescens* being member of Enterobacteriaceae family can be differentiated from other species by producing surfactant sarawettin, different marcescens, and extracellular proteases. S. *marcescens* extracellular proteases were shown to be toxic to an insect . These bacteria inhabit *Bombyx mori* which helps in digestion of the ingested food material by conglomerating with amylase, caseinase, gelatinase, lipase and urease . *Serratia Sp.*, adhere to the substratum with capsule, pili and utilize organic and inorganic substrates as food based on availability; it survives at different temperature and pH range so adapts to diversified environment. *Serratia marcescens* is normally considered to be opportunistic or facultative pathogen, but it is avirulent to insects when present in the digestive tract but becomes lethal on entering insect hemocoel following injury or stress. Serratia more often found in insectaries due to distribution of epizootics in air, water, or diet ingredients, it persists and grow in the guts of

insects of healthy and diseased ones (Bucher 1963, Sri-Arunotai et al. 1975); epizootics of *Serratia* in fecal contamination bacterium may be transmitted trans ovum .

Materials and Methods

Mulberry silkworm races of high yielding variety were reared in study area Victory-1','S','Kolar Gold' (Multi-voltine), Poly hybrids (Bi-voltine),among them Kolar gold (multivoltine) extensively reared, among them 5th instar larvae were obtained from Silk rearing farmers for mulberry feeding experiment. Mulberry leaves for isolation of septicemia bacteria and for feeding experiment were obtained from Mulberry growing fields in study area. Diseased Silkworms of septicemia like infection were got from silk rearing farmers.

Isolation of bacterium

Isolation of septicemia causing bacterium on silkworm was carried out in a silk rearing environment using samples of soil, phylloplane, diseased silkworm and rearing house. Samples collected from different parts of study area were subjected to isolation by following procedures of serial dilution 0.2, 0.4, and 0.6ml aliquots were cultured by pour plate method on nutrient agar and incubated for 72-96 hrs at 30^{o}c. As the dilution rate increases organism of interest may or may not isolate thus samples of lower dilutions were plated onto minimal media. Identifying isolates on the basis of cultural and biochemical characteristics gives insights about the transfer of pathogen from source to silk worm larvae.

The isolation of bacteria from silk worm rearing samples were carried in following methods from: a) **Soil sample:** Mulberry plants rhizosphere soil collected from different sampling units of study area (Chickkabalpura, Ramnagar and Kolar districts in Karnataka) using polythene covers of 0.4µ in different seasons rainy, summer and winter. Soil samples collected were serially diluted 0.2, 0.4 and 0.6ml aliquots of dilution grown on nutrient agar media (20ml /plate) by the pour plate method and incubated at 30± 2°C. Red pigment producing organism was found growing on an incubated plate as a mixed colony, pure colony obtained by streaking and restreaking onto nutrient agar plates. Preliminary identification of bacteria was based on pigmentation on plates after 3-4 days incubation.**b) Silkworm rearing trays**: The autoclaved cotton swabs were used for swabbing on trays later cultured onto nutrient agar by spread plate method to obtain bacteria of interest. The cotton swabs were immersed in sterile water for preparing the stock solution later serially diluted and cultured by pour plate method and incubated at 30±2°c for 3-4 days. **c) Phylloplane:** Samples of phylloplane were collected by washing mulberry leaf with sterile water; later serially diluted samples were plated onto nutrient agar and scrutinized for *Serratia* species. The cotton swabbing method was also used for swabbing on the phylloplane later cultured onto nutrient agar by spread plate method to obtain phylloplane bacteria.**d) Diseased silkworm:** Diseased dead and live silkworm larvae with known symptoms were collected. Live larvae anaesthetized and surface sterilized with 70% alcohol, haemocoel of larvae were collected by piercing larvae skin through the sterilized needle and is serially diluted using distilled water, plated onto nutrient agar and incubated for 72 - 96 hrs.**e)Cultures from walls:**There was destruction of vegetative forms by using chemical disinfectants in surveyed area, so also fungal spores remained on the walls as these can thrive on paints by using them as alternate source food. Isolation was carried out by taking samples later diluted serially and incubated for 5-7 days at 37° c.

Effect of soil isolate on silkworm

Silkworm mulberry leaf feeding experiment was done to know septicemia causing ability of bacteria from infection source leaves to haemocoel through the gut. The leaves were surface-sterilized in a 0.5% sodium hypochlorite solution for 10 min and washed for 2 h in water by in tap water containing tray followed by a final 5 min wash in sterile distilled water. Septicemia causing bacterial cultures of 24 hrs *Bacillus cereus, Serratia marcescens* and soil isolate suspension of 0.2,0.4 and 0.6 dilutions were smeared allowed to air dry for 30 min (Inglis & Lawrence, 2001; Sikorowski, 1998a)Larvae of 5th instar were placed in isolation with air dried leaf; larvae that did not consume the leaf during the period were excluded from the test. Smeared mulberry leaves fed to starving placing 5th instar larvatill cocoon formation, during period of observation larvae showed no indication of the appearance of symptoms like septicemia of silkworm.

Silkworm injecting experiments conducted to know soil isolate can cause septicemia on injecting into haemocoel. To compare and contrast between known septicemia causing strains such as *Bacillus cereus* and *Serratia marcescens,* (obtained from the culture collection centre NICL, Mumbai) with soil isolate were tested separately by injecting into the haemocoel of 5th instar silkworm larvae using a micro syringe with different aliquots of 0.2, 0.4 and 0.6 and larvae were kept under observation.Preliminary identification of bacteria in the haemocoel done by observing colour changes in the haemocoel of silkworm caused due to septicemia. Experimented septicemia causing bacteria and unknown isolate of soil were checked for Koch postulates. Even though preliminary observation of injected experiment showed haemocoel to colour change, soil isolate could not be confirmed as a

member of *Serratia*, so it became necessary to use methods of morphological and biochemical characterization to confirm soil isolate as *Serratia* species.

Biochemical characterization

Isolated organism was differentiated using gram staining, later they are biochemically characterized as per Bergy's Manual of Determinative Bacteriology' were performed on 48 hrs grown bacterial cultures, using standard protocols such as Catalase, lipase and oxidase production , glucose utilization tests, , iii) Simons citrate utilization and nitrate reduction , hydrolysis of cellulose ,and gelatin, and Methyl red, V P, Urease, H2S as per 'Bergey's Manual of Determinative Bacteriology.

Results

The results of experiments of isolation, and effect of soil isolate on injection, biochemical characterization after preliminary identification is given below in brief. The bacterial, fungal and actinomycetes colonies on agar plate were found to be numerous in rhizosphere soil sample of rainy season (fig1a and Table I). Numerous kinds of different magnitude micro organisms were found, as dilutions increased number of microbial colonies was reduced. During winter, available water and exudates of plant were low so cultured plates contained few bacterial and high densities of fungal colonies due to sporulation (fig 1b and Table II). During summer available water was low, so culture plates of soil inoculate showed the less number of bacterial and fungal colonies and there was no indication of the appearance of actinomycetes (shown in Table III and fig1c). Samples of silkworm rearing room walls and tray samples cultured on nutrient agar plates showed the less number of bacterial and fungal colonies, however bacteria on trays and rearing appliances were found so growth occurred in plates, however in less numbers.Diseased silkworm haemocoel was alkaline so the less number of bacteria and fungi found in haemocoel as well as in cultured plates, among bacteria in plates *Serratia* or its species were found to be absent (fig 1d,e,f and Table VI). Absence of *Serratia sp.,* and fungal colonies in cultured plates of the diseased silkworm sample indicated fungi cannot enter through silkworm protective layers and *Serratia* cannot sustain in high alkaline pH of haemocoel of silkworm. Bacteria and fungi were found negligible in cultured plates as aerosols are source phylloplane, during winter number of fungal colonies were found to be high as phylloplane will not provide a conducive environment for the proliferation of bacteria (fig 1d,eandTable VII).

In leaf feeding experiment we observed that bacteria smeared leaves on ingestion showed no sign of entering into the haemocoel thus indicating bacteria cannot enter from the digestive tract to haemocoel even though administered through food. In injection experiment, red bacteria isolated from mulberry rhizosphere soil, was injected into silkworm, interaction between bacteria and silkworm lead to following symptoms in silkworm similar to Silkworm septicemial symptoms caused by known septicemia bacteria, such as stopped eating mulberry leaves, hiding dorsal side of leaves, the movements became sluggish, thorax swelled, vomiting and excretion of soft waste occurred, body turned soft elongated and discolored, extended head and thorax, histolysis leading to accumulation of foul-smelling liquid, and finally death of silkworm similar symptoms are shown from previous observations of (Tao et al., 2011). Soil isolate secreted crimson red pigment so corpse of experimented silkworms contained orange fluid, fluid later turning to black in color same as that of septicemial infection.

Results of biochemical tests conducted to characterize soil isolate with *Serratia marcescens* and *Bacillus cereus* are summarized in Table 8. The tested strain showed positive results for Gelatin liquification, Nitrate reduction test, cellulose degradation test, Catalase test, Lipid hydrolysis test, carbohydrate utilisation test (dextrose), Citrate test and Methyl red test. The results obtained in our laboratory for soil isolate were consistent with the results of Bergy's Manual of Determinative Bacteriology (Holt and Krieg, 1994). Production of casienase enzyme by *Serratia sp.,* showing clear zone in plate (fig 14) around inoculation differentiated it from septicaemia bacteria.According to reports in the literature, morphological and biochemical experiments with Serratia and soil isolate yielded higher similarity values confirming soil isolate belongs to genus Serratia. However, strains belonging to *Serratia marcescens* differed with those of soil isolate in terms of some biochemical characteristics such as Indole test and Voges-Proskauer test. These reactions differentiated *Serratia plymuthica* and *Serratia marcescens*our results agree with Fischer's descriptions (1987) which differentiate between *S. plymuthica* and *S. marcescens*.

Table I: From soil in rainy season

Dilutions in ml.	Types of colonies			Grams staining	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	+++	+++	++++	+	+
0.4	++	+++	++	+	+
0.6	++	+++	+	+	+

TableII: From soil in winter season

Dilutions in ml.	Types and no. of colonies			Grams staining	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	++++	++++	++++	+	+
0.4	+++	++++	+++	+	+
0.6	+	++++	+	+	+

Table III: In summer season

Dilutions in ml.	Types of colonies			Grams reaction	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	1-2	+++	+++	+	+
0.4	_	+++	++	+	+
0.6	_	+	_	+	+

Table IV: Isolates from trays

Dilutions in ml.	Types of colonies			Grams reaction	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	++	++++	_	+	+
0.4	_	+++	_	+	+
0.6	_	+++	_	+	+

Table V: Isolates from walls

Dilutions in ml.	Types of colonies			Grams staining	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	++++	+	+++	+	+
0.4	++++	+	+++	+	+
0.6	++	+	_	+	+

Table VI: Isolates from infected silk worm

Dilutions in ml	Types of colonies			Grams staining	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	_	++	_	+	+
0.4	_	++	_	+	+
0.6	_	++	_	+	+

Table VII: Isolates from Phylloplane

Dilutions in ml	Types of colonies			Grams staining	
	Fungi	Bacteria	Others	Gram +ve	Gram –ve
0.2	++	++	_	+	+
0.4	_	++	_	+	+
0.6	_	++	_	+	+

+++ indicates presence of many colonies.
++ indicates presence of few colonies.
+ indicates presence of 1-2 colonies/
gram positiveand gram negative bacteria.
- indicates absence colonies.

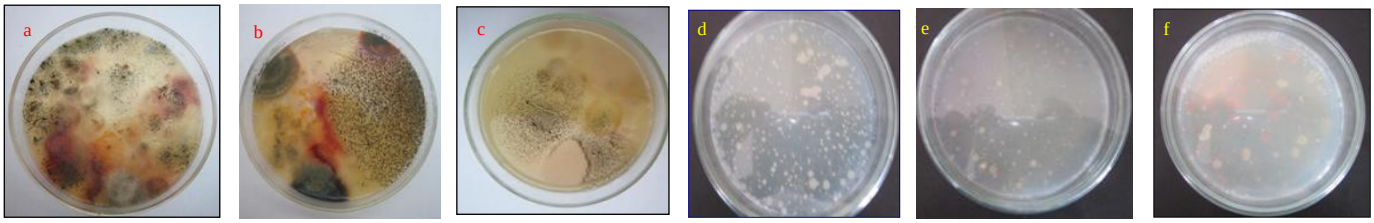

Fig 1: Nutrient agar (NA) plate of 0.4ml dilution of soil sample showing high density of microbial growth after 7 days of incubation at 30°±2ºc a) in rainy season, b) winter season and c) summer season.
Fig 1d: NA plate of 0.6ml dilution ofdiseased silkworm sample showing bacterial growth after incubation at 30º±2ºc

for 4days.

Fig 1e: NA plate of 0.6 ml dilution of rearing appliances sample showing bacterial growth after incubation at 30º±2ºc for 4days.

Fig 1f: NA plate showing growth of red pigmented bacteria from soil sample at o.6 mldilution.

Table 8: Biochemical Tests todeterminemicrobial characteristics

Sl. No.	Biochemical tests	Experimented microorganisms	Septicemia causing	
		Serratia marscenes	*Bacillus cereus*	Unknown soil isolate
1.	Indole	-	-	-
2.	Methylred	+	+	+
3.	Vougesproskauer	-	-	+
4.	Citrate	+	+	+
5.	Oxidase	-	+	-
6.	Urea hydrolysis	+	+	+
7.	Hydrogen sulphide	-	-	-
8.	Gelatin hydrolysis	+	+	+
9.	Nitrate reduction	+	+	+
10.	Cellulose hydrolysis	+	+	+
11.	Catalase	+	+	+
12.	Casien hydrolysis	+	-	+
13.	Lipid hydrolysis	+	+	+
14.	Carbohydrate (dextrose)	+	+	+

\+ Sign indicates presence of activity

\- Sign indicates absence of activity

Discussions

Multivoltine mulberry silkworm larvae having a growth period of a month produces silk is used in economically productive silk rearing activity. Most of pre and post losses to economy of silk industry is attributed to silkworm diseases caused by different microbial agents among them bacteria play an important role as causal organism of disease. Among bacteria Enterobacteriaceae family plays a major role in causing disease to *Bombyx mori* in different stages of its life cycle. However larval stage is susceptible and prone to many diseases. Hence it is important to identify sources and pathogenicity of unknown bacteria to dwindle losses caused by bacterial diseases to the silk industry. Most of the pathogenic interaction between insects and entomopathogenic bacteria are caused due to loss of natural immunity of insects. The identification of pathogens and understanding of biochemical changes that appear during infection in silkworm due to infection will upgrade naval methods of disinfecting diseases and to make disease resistant varieties of silkworm.

The Isolation of septicemia causing *Serratia sp.,* was carried out using samples of soil, phylloplane, diseased silkworm and rearing house in our study area.The results obtained have shown that rhizosphere soil in rainy season contains high diversity and density of microbes (fungi, bacteria and actinomycetes) due to high availability of moisture, exudates of roots, and supplementary organic and inorganic matter in rhizosphere soil. Fungi, bacteria and actinomycetes do not produce any clear zones in the media by secreting secondary metabolites after incubating for7 days also,indicating these microbes as commensals.Above conducible conditions were persistent in rainy season, so active multiplication of microbes occurred but bacteria and fungi rarely entered into a dormant sporulation stage, spores formed does not retain dormancy. Aeration of soil in pre-rainy season provided high oxygen in soil for proliferation of actinomycetes in soil. In rainy season rhizosphere soil showed lower no.of red coloured, gram negative bacteria indicating a growth rate of organisms was high in comparison to other seasons. In winter available water and exudates of plant were lower than rainy season, so led to decrease in bacterial multiplication rate. Sporulation in fungal species was hastened by the onset of unfavourable conditions as vegetative forms cannot survive thus spores formation, other microbial species like actinomycetes absent in plates as aeration of soil was low.During summer season there were less No.of microbial colonies due to low water availability. Silkworm rearing room walls and trays were maintained in good hygienic condition, so bacterial and fungal species were found in low numbers on culturing their samples.Even though there was prevalent pre-disinfectant method applied, sampled walls

contained fungal spores, as spores remain dormant on returning to vegetative stage can thrive on paints by using paint as an alternate source of food. Bacteria on trays and rearing appliances were found to be common, so occurred in plates however in less numbers. Mulberry leaves were subjected to different types of constraints like wind, rain, high intensity of light thus the less number of bacteria and fungi isolated from leaf samples, so also lacking septicemia causing organism in the sample. Diseased silkworm cultured plates showed occurrence of bacteria without fungal growth indicating that fungal species can grow only on the surface of larval skin and cannot enter through skin into haemocoel of larvae.

Studies conducted to know soil isolate belong to *Serratia* species or other red metabolite prodigiosin producing bacteria.There found numerous red colored bacterial strains *Vibrio psychroerythrus, Streptomyces coelicolor*, *Pseudomonas magnesiorubra*, *Streptomyces lividans,Alteromonas rubra*, *Streptomyces spp*., *Hahella chejuensi*, *Pseudovibrio denitriccans*, *Pseudoalteromonas rubra* and Nocardia spp (Chellappan Sumathi et al., 2012) found in nature thus creating confusion during characterization of red bacterium *Serratia* species. Some of *Serratia sp.*, can cause septicaemia, so soil isolate tested for ability to cause septicaemia in silkworm showed positive results and to confirm soil isolate belongs to *Serratia sp.*, cultural and biochemical characters with known septicemia causing *Bacillus SP.*, and *Serratia sp.*, (obtained from the culture collection centre NICL, Mumbai) were carried out. Most of multivoltine strains of silkworm larvae use mulberry leaves as their food, which may act as source of bacterial septicemia, so mulberry leaves were subjected to microbial testing but no septicemia agents were found in leaves indicating leaves were not means of septicemia in silkworm.

Rhizosphere soil isolates were examined for septicemia development in silkworm by leaf feeding experiment. The bacteria does not enter into the haemocoel through gut which was proved in our infected leaf feeding experiment, ,but bacteria can enter into larval haemocoel through wounds caused by stress or injury indicated in previous work. The study of injecting soil isolate bacteria was carried out on 5^{th} instar larvae of silkworm, larvae showed no significant difference in symptoms between septicemia causing agents and soil isolate thus indicating soil isolate is one among septicemial agent. Further confirmation through cultural and biochemical characterization indicated soil isolate is one among *Serratia sp*., but few deviations in biochemical characters with *Serratia marcescens* strain indicate, it may be different strain but belongs to similar septicemia causing *Serratia* species. The ability to cause septicemia by soil isolate was confirmed by testing it with Koch postulates in relation to known septicemia causing organism. Biochemical characterization of soil isolate deviated from *Serratia marscenes* by producing acetoin a precursor of 2, 3 butanediol and ethanol, but known septicaemia causing experimented *Serratia marscenes* and *Bacillus cereus* does not produced this product. The absence of cytochrome oxidase in *Serratia marscenes* and soil isolate with other similar characteristics like gram negative, motile with pritrichous flagella, prodiogisin pigment producing and other biochemical characters indicates these belong to same genus. The key experimental results show that soil isolate is found to be *Serratia plymuthica*. The insights of our study will ease the way in prevention and cure of Silkworm septicemia disease caused by soil isolate as pathogen to silkworm.

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