MICROBIAL FLORA IN GUT OF *CULEX QUINQUEFASCIATUS* BREEDING IN CESS PITS

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Abstract. The number and types of microorganisms in the gut of *Culex quinquefasciatus* larvae varied considerably from one site of collection to another. Larval gut, in general, contained enormous number of bacteria, a few fungi and negligible number of actinomycetes which belonged to 15 bacterial, 6 fungal and 4 actinomycete genera, respectively. *Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas* sp. among bacteria, *Aspergillus* among fungi and *Streptomyces* sp. among actinomycetes were frequently encountered. *Escherichia, Proteus, Aspergillus* and *Streptomyces* were the most abundant genera. Isolates of *Bacillus, Pseudomonas, Shigella* and *Staphylococcus* caused 100% mortality during the early instar of larval development. None of the fungal isolates effected 100% mortality while *Nocardiopsis* sp. among actinomycetes gave 100% mortality. One of the *Escherichia* isolate suppressed the adult emergence completely while 27 others, belonging to most of the genera found, suppressed significantly. Isolates of *Aspergillus, Alternaria* and *Streptomyces* inhibited the emergence of adults completely.

INTRODUCTION

Considerable knowledge has accumulated regarding microorganisms as parasites of mosquitos and mosquitos as vectors of pathogenic microorganisms. But knowledge of the relationship of microorganisms to mosquitos *per se* is scanty. To generate such information the prerequisite is data on the gut flora of mosquitos which also is scanty (Lysenko, 1985). In spite of the potential, when seen from the viewpoint of microorganisms as biocontrol agents, this aspect has failed to attract much attention; even the recent cloning of genes of toxins of mosquito pathogenic spore-forming bacilli (Baumann *et al,* 1988; Whiteley *et al,* 1988) has failed to do so. Because of this latter advance, knowledge of the gut flora of mosquitos has become more meaningful in terms of the practical implications; further data would also help in understanding the relationship between microflora and larval development.

Larvae of *Culex quinquefasciatus*, the vector of bancroftian filariasis, mainly inhabit highly polluted habitats like cess pits and drains. These habitats are the receptacles of sullage water from households. The sullage water, being highly polluted, is likely to harbor abundant microorganisms. Due to the filter feeding behavior of the larvae, these microorganisms will enter the larval gut. Information on the nature of these microorganisms and their interaction with *Cx. quinquefasciatus* larvae are not readily available. Hence a study was undertaken to quantify the gut microflora of these larvae and understand their influence on larval growth and developments.

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MATERIALS AND METHODS

Sample collection and processing

Larval samples were collected from 10 cess pits located in different areas of Pondicherry, viz Kadirgamam, Kottakuppam, Mudaliarpet, Muthialpet, Vanarpet and Jeevananthapuram. Using a 250 ml capacity disinfected dipper, five samples were taken from the 4 corners and the center of the pit. Fifty healthy looking III instar larvae were picked up with a sterile pasteur pipet and transferred to a sterile 250 ml screw capped

bottle along with ~50 ml of cess pit water. The samples were brought to the laboratory and processed immediately.

The larvae were removed from the field water and the oral region, anus and siphon of each larva were sealed with sterile paraffin. Then the larvae were washed twice with sterile distilled water, surface sterilized with 0.1% mercuric chloride and again washed twice with sterile distilled water. The midguts of the larvae were drawn out gently and placed in 2 ml sterile saline (0.85%) in a sterile glass tissue homogenizer. They were homogenized for two minutes. The pestle of the homogenizer was washed again with 1 ml sterile saline in the same tube. All operations were carried out under aseptic conditions.

Isolation of microorganisms

Serial dilutions of the gut homogenates were made in sterile saline, plated on BHI agar medium (Anonymous, 1977) for the isolation of bacteria, Richards's medium (Bilgrami *et al,* 1981) for fungi and YMG agar medium (Bilgrami *et al,* 1981) for fungi and YMG agar medium (Lapage *et al,* 1970) for actinomycetes. These plates were incubated at 30 ± 2°C for 24 hours, 5-7 days and for 5-10 days for observing the development of bacterial, fungal and actinomycete colonies, respectively.

No attempt was made to culture obligate anaerobes. After the completion of the incubation period the different colony types which appeared on the plates were counted and recorded. Then each type of colony was purified, pure cultures were subcultured and maintained on respective media and when necessary stored at 4°C until use.

Identification of isolates

The procedures followed in the determination of bacterial genera was based on the methods described by Buchnan and Gibbons (1974). The fungal and actinomycete isolated were identified based on colony and cell characteristics (Rippon, 1988; Williams *et al,* 1989). The slide culture technique was employed to study the morphology of actinomycetes.

Effect of gut flora on the development of mosquito larvae

To study the effect of gut micro-flora on the growth and development of *Cx. quinquefasciatus,* the larvae were reared from first instar to the adult stage in the presence of individual gut microorganism as follows. Each bacterial isolate was inoculated into 10 ml BHI broth in a boiling tube and incubated on a rotary shaker at 30 ± 2°C for 48 hours. Fungal and actinomycete isolates were grown in Richard's broth and YMG broth, respectively, and incubated at room temperature for 20 days, as stationary cultures. Then 1 ml of broth culture was administered to 50 *Cx. quinquefasciatus* larvae (from laboratory colony) held in 249 ml of deionized water in a 500 ml capacity sterile beaker. The tests were run in quardruplicate along with appropriate controls. 10 ml liquid larval food (5% suspension of a mixture of yeast and dog buiscuit powder at 1:2 ratio) was added to each beaker every day. The beakers were held at room temperature (30 ± 2°C), covered with mosquito net cloth to prevent the entry of other insects and escape of emerged adults. The water loss in the beakers due to evaporation was compensated with sterile deionized water. The larvae were observed daily for the number of immatures surviving successive stages of development and numbers of adults emerging. Whenever larval mortality was observed the percent mortality was calculated using probit analysis, after correcting for control mortality, if any, using Abbot's formula (1925). The experiment was terminated when all the larvae in controls emerged as adults.

RESULTS AND DISCUSSION

Occurence of microorganisms in the gut

The number of colony forming units (CFU) of bacteria, fungi and actinomycetes obtained from guts of *Cx. quinquefasciatus* larvae collected from different sites are shown in Fig 1. The total number of different organisms from 50 guts of larvae collected from different sites ranged from 3×10^3 - 5×10^5 CFUs, 7×10^1 - 1×10^4 and 0 - 60×10^1, respectively. The larvae from site No. 5 had the highest number of bacteria (5×10^5 CFUs/50 guts) but least number of fungi (6.9×10^1 CFUs/50guts) and no actinomycetes. On the other hand, larvae

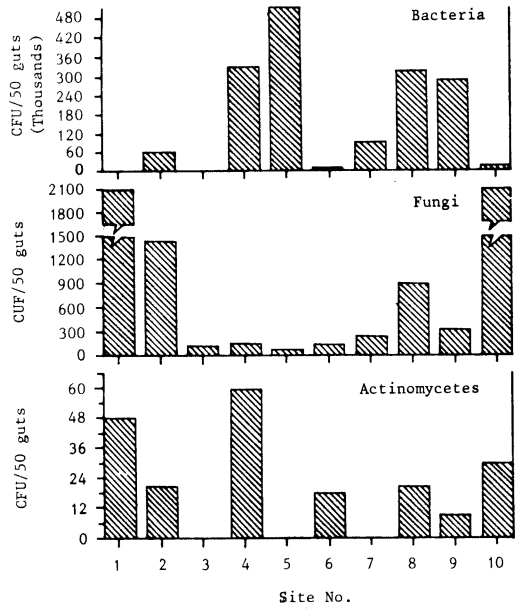

Fig 1—The abundance of microorganisms in different sites.
1. Kadirgammam, 2. Kottakuppam I, 3. Kottakuppam II, 4. Muthialpet, 5. Vanerpet I, 6. Vanerpet II, 7. Mudaliarpet I, 8. Mudaliarpet II, 9. Jeevanandhapuram I, 10. Jeevanandhapuram II.

from site No.1 contained very few bacteria (3×10^3 CFU/50 guts) and a greater number of fungi (1×10^4) and actinomycetes (4.8×10^1 CFUs). Larvae from site No.8 contained all the three types of organisms in good numbers. On an average, 1.6×10^4 bacteria, 17×10^2 fungal and 2.07×10^1 actinomycetes CFUs were obtained per 50 larval gut. This works out to 3×10^3 bacterial, 3.4×10^1 fungal and 4×10^{-1} actinomycete CFUs/larval gut and the proportion of bacteria, fungi and actinomycetes in a gut works out at 98.95, 1 and 0.01% of the total number of microorganisms respectively. The results indicate that the number of different types of microorganisms in the larval gut vary considerably, from one site to another and that larval guts in general contain enormous numbers of bacteria, a few fungi and a negligible number of actinomycetes.

Among the gut flora encountered there were 54 types of bacteria, 47 types of fungi and 10 types of actinomycetes. On identification the bacterial isolates were found to belong to 15 genera, fungal isolates to 6 genera and actinomycetes to 4 genera (Table 1).

The frequency and average counts of different genera of bacteria, fungi and actinomycetes are presented in Fig 2, 3 and 4. Among bacteria *Bacillus, Staphylococcus* and *Pseudomonas,* among fungi, *Aspergillus* and among actinomycetes, *Streptomyces* were more frequently encountered. *Escherichia* and *Proteus* were found to occur in higher number (2×10^5 and 1.7×10^5/50 guts, respectively) followed by *Bacillus, Staphylococcus,*

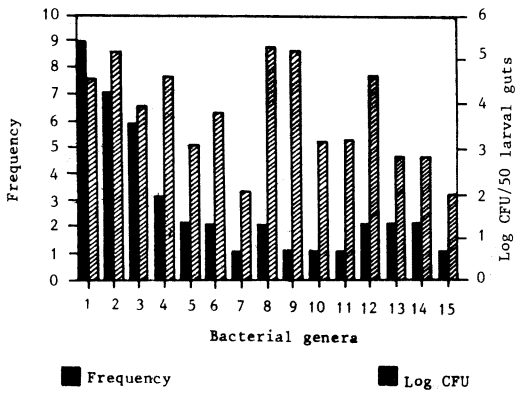

Fig 2—The frequency and average counts (CFU) of different genera of bacteria in the guts of *Cx. quinquefasciatus* larvae from cess pits.
1. *Bacillus* sp, 2. *Staphylococcus* sp, 3. *Pseudomonas* sp, 4. *Serratia* sp, 5. *Shigella* sp, 6. *Flavobacterium* sp, 7. *Listeria* sp, 8. *Escherichia* sp, 12. *Enterobacter* sp, 13. *Pectobacterium* sp, 14. *Salmonella* sp, 15. *Micrococcus* sp.

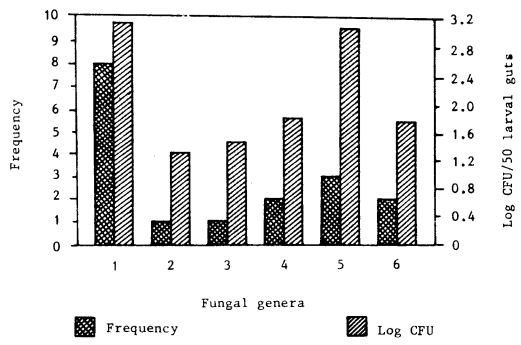

Fig 3—The frequency and average counts (CFU) of different fungi in the guts of *Cx. quinquefasciatus* larvae from cess pits.
1. *Aspergillus* sp, 2. *Pullularia* sp, 3. *Alternaria* sp, 4. *Fusarium* sp, 5. *Microsporum* sp, 6. *Heterosporium* sp.

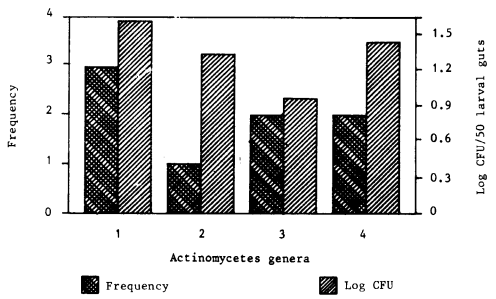

Fig 4—The frequency and average counts (CFU) of different genera of actinomycetes in the guts of *Cx. quinquefasciatus* larvae from cess pits.
1. *Streptomyces* sp, 2. *Micromonospora* sp, 3. *Nocardiopsis* sp, 4. *Nocardia* sp.

Serratia and *Enterobacter* (1.5×10^4 to 4.5×10^4). Other genera occurred in comparatively low numbers (1×10^2 to 8.8×10^3). Among fungi aspergilli were highly abundant in the larval guts (1.5×10^3), compared to other genera viz, *Fusarium, Heterosporium, Pullularia* and *Alternaria* (2.1×10^1-6.4×10^1). *Streptomyces* were more abundant (3.9×10^1) among the actinomycetes followed by *Nocardia, Micromonospora* and *Nocardiopsis* ($0.9 - 2.5 \times 10^1$).

Cx. quinquefasciatus larvae, being filter feeders, ingest any small particle, including microorganisms, that enters the vortex set up by mouth brushes. Chao and Wistreich (1960) have found *Bacillus* and *Saccharomyces* in the guts of larvae obtained from laboratory colonies. In the present study bacteria predominated the larval gut of *Cx. quinquefasciatus* larvae collected from cess pits, while fungi and actinomycetes formed very small proportions. *Bacillus, Staphylococcus* and *Pseudomonas* among bacteria and aspergilli among fungi were more abundant.

All the bacterial, fungal and actinomycete isolates were tested for their effect on the development of *Cx. quinquefasciatus* larvae. In the control set of experiments it took 9 days for 1st instars to become adults. And with 7% natural mortality at fourth instar stage all the survivors pupated and emerged (Table 1).

In the treated sets, 54 of the 57 bacterial isolates caused larval mortality at different levels. The mortality was 100% with 14 isolates and they belonged to genera *Bacillus, Pseudomonas, Shigella* and *Staphylococcus* (Table 1). However, none of the *Bacillus* isolates were either *Bacillus thuringiensis* and *B. sphaericus* which are well known as larvicides (Anonymous 1979, 1980). Of the remaining isolates, 30 effected 51-90% mortality and 9 1-50% mortality. *Bacillus* and *Pseudomonas* species have frequently been isolated as facultative insect pathogens (Bulla *et al*, 1975; Mishra *et al*, 1987), while *Serratia, Enterobacter, Klebsiella, Proteus, Alcaligenes, Flavobacterium* species have been isolated as opportunistic insect pathogens (Lysenko, 1985). The observations of the present study support these findings wherein *Bacillus, Pseudomonas, Shigella, Staphylococcus, Flavobacterium, Serratia, Enterobacter, Alcaligenes, Pectobacterium, Acinetobacter, Escherichia, Listeria, Proteus, Micrococcus* and *Salmonella* isolated from the larval gut have been found to cause larval mortality.

All the 47 fungal isolates caused larval mortality. With 19 isolates the mortality was more than 50% and the majority of these belonged to the genus *Aspergillus.* The remaining isolates caused 5.0-50% mortality. Among actinomycetes, one isolate of *Nocardiopsis* sp. caused 100% mortality and an other less than 50% mortality. The rest caused 51-99% mortality (Table 1). Fungi and actinomycetes are known to produce several metabolites active against insects (Ando, 1982; Misato, 1982). Avermectins produced by *Streptomyces avermitilis* is one among these (Burg *et al*, 1979). Similarly, actinomycetes such as *Streptomyces, Actinoplanes, Actinomadura, Streptoverticillium, Micromonospora, Micropolyspora, Nocardiopsis, Streptosporangium* and *Thermonospora* and fungi belonging to genera *Aspergillus. Penicillium, Monilia* and *Fusarium* have been reported to produce toxic metabolites against mosquito larvae (Mishra *et al*, 1987). *Alternaria, Verticillium, Cephalosporium, Botrytis, Chaetomium, Penicillium, Trichoderma, Hormodendrium, Metarrhizium anisopliae, Beauveria bassiana* have also been reported to be larvicidal by other workers (Denebekov *et al*, 1977; Mishra *et al*, 1987; Balaraman *et al*, 1979). In the present study fungi belonging to *Alternaria, Aspergillus, Fusarium, Heterosporium, Microsporum* and *Pullularia* and actimonycetes belonging to *Micromonospora, Nocardia, Nocardiopsis* and *Streptomyces* obtained from the larval gut were found to be larvicidal.

Table 1

Effect of gut micro-flora on *Culex quinquefasciatus* larvae.

Genus	Total No. isolates	Isolates exhibiting					
		Mortality (%)			Suppression of emergence (%)		
		1-50	51-99	100	1-50	51-99	100
Bacteria							
Alcaligenes	1		1			1	
Acinetobacter	1		1			1	
Bacillus	16	2	8	6	4	6	(6)
Enterobacter	2		2		1	1	
Escherichia	2		2			1	1
Flavobacterium	2	1	1		1	1	
Listeria	1	1				1	
Micrococcus	1		1			1	
Pectobacterium	4		4		2	2	
Pseudomonas	7	2		5	2		(5)
Proteus	1		1		1		
Salmonella		2			1	1	
Serratia	4	1	3		3	1	
Shigella	2		1	1		1	(1)
Staphylococcus	7		5	2	2	3	(2)
Fungi							
Alternaria	1	1			1		
Aspergillus	37	25	12		12	25	
Fusarium	2		2		2		
Heterosporium	2		2		1	1	
Microsporum	4	2	2		1	3	
Pullularia	1		1			1	
Actinomycetes							
Micromonospora	1		1			(1)	
Nocardia	2		2		1	1	
Nocardiopsis	2	1		1		1	1
Streptomyces	5		5		1	3	1

Control - Mortality 6%, Suppression of Emergence 2%.
Figures in parenthesis indicate suppression due to mortality.

Inhibition of adult emergence

Among the 54 bacterial isolates 2 isolates belonging to genus *Escherichia* significantly affected the adult emergence. While one isolate caused 100% suppression of the emergence the other caused more than 50% suppression (Table 1). Of the remaining isolates nearly half of them (27) caused more than 50% suppression and the rest less than 50% suppression. Majority of the fungal isolates (30) caused 51-99% suppression in the emergence (Table 1). Isolates belonging to genera *Aspergillus* and *Alternaria* caused 100% suppression. Among the actinomycetes one of the *Streptomyces* isolates caused 100% suppression (Table 1). Five isolates belonging to genera *Micromonospora, Streptomyces* and *Nocardiopsis* caused 51-99% suppression. Sakuda *et al,* (1987) have reported

that a *Streptomyces* isolate produced a chitinase inhibitor, allosamizolin, which inhibited the pupation in *Bombyx mori*. In the present study also the metabolite(s) produced by some of the bacteria, fungi and actinomycetes isolated from the larval gut affected the developmental process of *Cx. quinquefasciatus* larvae.

The present study suggests that the gut of *Cx. quinquefasciatus* larvae harbor diverse microbial populations with potential of affecting the development of the larval stages. Thus they might form one of the biotic factors influencing the development and survival of *Cx. quinquefasciatus* in natural ecosystems.

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