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DEVELOPMENT OF A HIGH LEVEL OF RESISTANCE TO *BACILLUS SPHAERICUS* IN A FIELD POPULATION OF *CULEX QUINQUEFASCIATUS* FROM KOCHI, INDIA

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ABSTRACT. Field resistance to *Bacillus sphaericus* was observed in a population of *Culex quinquefasciatus* in Kochi, India, exposed to 35 rounds of spraying with a formulation of *B. sphaericus* 1593M over a 2-year period. Larvae from the sprayed area gave LC_{50} and LC_{90} values that were 146 and 180 times greater than corresponding values for a susceptible strain from an unsprayed locality. When the resistant strain was colonized in the laboratory and subjected to moderate selection pressure at each generation, resistance rapidly increased and by the 18th generation it was 6,223 and 31,325 times greater at the LC_{50} and LC_{90} levels in comparison with the susceptible strain. There were no significant differences among 6 susceptible strains tested. Tests were repeated and validated using the standard primary powder SPH88, *B. sphaericus* 2362. No cross resistance was observed against *B. thuringiensis* H-14.

INTRODUCTION

In recent years much work has gone into developing microbial agents for the control of disease vectors, of which *Bacillus sphaericus* has been one of the most promising. *Bacillus sphaericus* is now undergoing extensive field trials in Zaire (Karch et al. 1992), Cameroon (Hougard et al. 1993), France (Sinegre et al. 1993), Brazil (Regis et al. 1995), and India (Centre for Research in Medical Entomology [CRME], unpublished data). Before *B. sphaericus* is used operationally in mosquito control programs, the possibility of development of resistance to this agent must be studied. A few studies have been carried out in the laboratory to select strains of mosquitoes resistant to this bacillus. Rodcharoen and Mulla (1994) obtained 37-fold resistance at LC_{50} with *Culex quinquefasciatus* Say after 80 generations of selection pressure with a dose expected to produce 80% mortality. However, an extremely high level of resistance to *B. sphaericus* (100,000-fold increase in LC_{50} by the 12th generation) was obtained by Georghiou et al. (1992) in the laboratory using 94–98% selection pressure. There is also a preliminary report of low level field resistance to *B. sphaericus* in a population of *Cx. quinquefasciatus* in Brazil (M. H. Silva-Filha et al., unpublished data). In this paper we report the development of a relatively high level of resistance in a field population of *Cx. quinquefasciatus* in Kochi, Kerala, India, exposed to repeated applications of *B. sphaericus,* and the subsequent selection of a strain with a high level of resistance in the laboratory.

MATERIALS AND METHODS

Background: A field trial was carried out in an 8-km^2 area in Gandhinagar, comprising 3 residential localities, all with a high level of breeding of *Cx. quinquefasciatus.* The most productive larval habitats were concrete-lined box-shaped drains and unlined drains. Biocide-S, a microgel droplet formulation of *B. sphaericus* 1593M developed by Kunthala Jayaraman, Department of Biotechnology, Anna University, Madras, was sprayed at a target dose of 20 mg/ft.^{2} (0.22 g/m^2) in the breeding sites at 2-wk intervals using compression sprayers. Production of the formulation and treatment of breeding places was carried out by staff of Anna University; the CRME (Madurai) carried out the evaluation. Thirty-five rounds of spraying were conducted between February 1991 and March 1993, with suspension of spraying during the southwest monsoon period (June–July) due to heavy rainfall, and from January 14 to February 28, 1992, and again in April 1992 because of the lack of a supply of Biocide-S.

Good control of breeding was obtained between February and December 1991 (Mani 1992). However, after resumption of regular spraying in 1992 satisfactory control was never obtained and it was suspected that the poor results could be due to the development of resistance in the field. Samples of larvae were collected from the treated area as well as from 3 localities adjacent to the treated area, and 2 localities about 10 km distant. The field-collected larvae were transported to Madurai, tested for resistance, and colonized.

Bacterial strains: The bacterial strains used were: 1) *B. sphaericus* 1593M (Biocide-S) in a microgel droplet formulation and standard powders of 2) *B. sphaericus* 2362 (SPH-88) and 3) *B. thuringiensis* var. *israelensis* (IPS-82) obtained from J. F. Charles, Institut Pasteur, Paris.

Colonization of mosquitoes: Larvae from different localities in Kochi were brought to the

laboratory. A sample from each was identified as *Cx. quinquefasciatus* and another sample was subjected to bioassay. Cycling colonies of mosquitoes from unsprayed areas were maintained in 30 × 30 × 30-cm cages separately for individual localities and fed on chickens and soaked raisins. Individual generations of progeny of the strain from the sprayed area (Gandhinagar) were maintained separately after being subjected to selection pressure with *B. sphaericus* as described below. All possible precautions were taken to prevent cross-contamination of the strains, which included keeping resistant strains in a separate room and using separate pipettes. A cycling colony of *Cx. quinquefasciatus* from Madurai that has been maintained for more than 8 years in the laboratory was included in the studies.

Bioassay methodology: The bioassay procedure followed for *B. sphaericus* was essentially that recommended by the World Health Organization (1985). Initially, the standard used against the field-collected strains was a microgel droplet formulation of 1593M that had also been used in the field. A stock solution of 5 mg/liter or 110 mg/liter was prepared in deionized water and thoroughly mixed by vigorous shaking by hand. Serial 2-fold dilutions from stock solutions (5 mg/liter for unsprayed areas and 110 mg/liter for sprayed areas) were prepared. Three replicates for each dilution and 25 late 3rd-instar larvae per replicate were used, and parallel controls were maintained. Larval food was provided and mortality was recorded at 48 h.

Subsequently, all the strains were reassayed for susceptibility to the standard *B. sphaericus* (SPH-88). A stock solution of 50 mg in 10 ml deionized water was prepared to which sterile glass beads were added and homogenized on a mechanical shaker. Two-fold dilutions were prepared from the stock solution and 5 replicates, each of 20 late 3rd-instar larvae, were exposed to each dilution; controls without treatment were also set up. The dosages tested ranged from 0.003 to 0.08 mg/liter for the strains from unsprayed areas and 5 to 300 mg/liter for the strains from the sprayed area.

Susceptibility tests were also carried out with the strain from the sprayed area to study whether the larvae developed cross-resistance to *B. thuringiensis* var. *israelensis* (IPS-82). The bioassay was carried out according to the World Health Organization's protocol (1981). A stock solution was prepared by adding 10 ml deionized water to 50 mg powder and homogenized as previously on a mechanical shaker using sterilized glass beads. From the stock solution, dilutions were made to obtain dosages ranging from 0.0003 to 0.01 mg/liter and 5 replicates each of 20 late 3rd-instar larvae were exposed to each dilution. Larval food was not provided and mortality of the larvae was recorded at 24 h. The Madurai strain of *Cx. quinquefasciatus* was included as control in all the experiments.

Selection of a resistant strain: Late 3rd-instar larvae from Gandhinagar (sprayed area) were subjected to dosages of *B. sphaericus* 1593M ranging from 5 to 40 mg/liter to determine the LC_{50} value. The mortality ranged from 41 to 99%. The surviving larvae from this experiment were pooled, rinsed in deionized water, and reared to the next generation (F_1). Late 3rd-instar larvae of the F_1 generation were again subjected to different doses to determine the LC_{50}, and the survivors were reared to the F_2 generation. This process was continued for 7 generations. The dosages used gave mortality from 20 to 97% in different experiments. Subsequently, from the F_8 to the F_{17} generations selection was applied at a single dose that was expected to produce 75% mortality. The F_{18} generation was tested for susceptibility using the standard powder *B. sphaericus* 2362.

Data analysis: The LC_{50} and LC_{90} values were established by probit analysis, using a software package "ASSAY" (courtesy of C. F. Curtis, London School of Tropical Medicine and Hygiene, London). Comparison of LC_{50}, LC_{90}, and slope values was carried out using ANOVA followed by Duncan's Multiple Range Test (DMRT) using the SPSS/PC+ statistical package version 4.0.1 (SPSS Inc., Chicago, IL, USA, 1984–1990).

RESULTS

Bioassays were carried out using the *B. sphaericus* 1593M strain to test the susceptibility of the Madurai laboratory strain of *Cx. quinquefasciatus,* 5 strains from unsprayed areas in Kochi (3 close to the sprayed area, 2 from about 10 km away), and one from the sprayed area, Gandhinagar. The LC_{50} and LC_{90} values for the Madurai strain were 0.03 and 0.14 mg/liter, respectively, and did not differ significantly from LC_{50} and LC_{90} values for 4 strains from unsprayed areas in Kochi, which ranged from 0.007 to 0.17 mg/liter and from 0.02 to 0.42 mg/liter, respectively. The corresponding values of the 5th strain, Vytilla, were considerably higher (LC_{50} 0.36 mg/liter and LC_{90} 1.81 mg/liter) than those of other unsprayed localities and the Madurai laboratory strain. However, the fiducial limits of all the strains overlapped, and there was no significant difference at the 5% level using DMRT. The LC_{50} and LC_{90} values for the strain from Gandhinagar were 7.3 and 27.5 mg/liter, respectively, 146 and 180 times greater than the Fort Kochi strain, which was taken to represent the susceptible Kochi population, because it was well isolated from

Table 1. LC_{50} and LC_{90} values of *Culex quinquefasciatus* from unsprayed areas and areas sprayed with *Bacillus sphaericus* 1593M.

Area	LC_{50} (mg/liter)	95% fiducial limits Upper	95% fiducial limits Lower	LC_{90} (mg/liter)	95% fiducial limits Upper	95% fiducial limits Lower	Slope
1. Kallur	0.007a[1]	0.008	0.006	0.02a	0.03	0.019	2.48a
2. Madurai (Lab)	0.03a	0.04	0.03	0.14a	0.17	0.11	1.99a
3. Fort Kochi	0.05a	0.05	0.04	0.15a	0.18	0.12	2.64a
4. Mattancherry	0.08a	0.16	0.04	0.39a	1.20	0.12	1.90a
5. Indiranagar	0.17a	0.24	0.01	0.42a	0.81	0.21	3.29a
6. Vytilla	0.36a	1.03	0.12	1.81a	21.50	0.15	1.82a
7. Gandhinagar	7.33b	38.70	1.38	27.45bc	469.27	1.60	2.23a
F_1	33.18c	205.30	5.36	141.48bc	13,867.73	1.40	2.03a
F_2	23.93c	38.09	15.04	82.07bc	241.40	27.90	2.40b
F_3	15.54c	57.39	4.21	72.31b	1,505.77	3.47	1.92b
F_5	70.31d	75.52	65.46	141.35c	175.00	114.18	4.23c
F_7	76.69e	83.20	76.33	119.52bc	129.94	109.94	7.30b

[1] Values followed by a common letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

the sprayed area (Table 1). Fiducial limits in this case, though large, did not overlap with those of the susceptible strains and the Gandhinagar strain was also significantly different from all unsprayed localities in DMRT.

Bioassays were carried out to test the susceptibility of the F_1 to F_7 generations reared from larvae from Gandhinagar and subjected to selection pressure (Table 1). There was a rapid and significant increase in LC_{50} and LC_{90} values and values for the F_7 generation were 1,594 and 797 times, respectively, greater than values obtained from the Fort Kochi strain. After this for some generations bioassays were not carried out because of shortage of the biopesticide. Variability (as shown by the wide 95% fiducial limits) was very high in the resistant strain in comparison with susceptible strains from unsprayed areas. No clear trends in slope values were observed.

All the strains were reassayed with a standard powder of *B. sphaericus* 2362 (SPH-88), which was received about 6 months later. As shown in Table 2 the Madurai laboratory colony was the most susceptible, with LC_{50} and LC_{90} values of 0.004 and 0.024 mg/liter, respectively. The LC_{50} and LC_{90} values for 4 susceptible strains from Kochi ranged from 0.008 to 0.015 mg/liter and from 0.028 to 0.085 mg/liter, and the Vytilla strain showed the highest values of 0.070 and 0.6 mg/liter, respectively. The 95% fiducial limits for the Vytilla strain did not overlap with any other strain but it did not differ significantly from other unsprayed areas by DMRT.

The 18th generation of the resistant strain (Gandhinagar), when bioassayed with *B. sphaericus* 2362, had LC_{50} and LC_{90} values of 49.78 and 971.07 mg/liter, respectively, 6,223 and 31,325 times greater than the values for the Fort Kochi strain. These differences were statistically significant. The slopes for the Gandhinagar and Vytilla strains were significantly less steep than those for the other unsprayed localities.

To test whether any cross-resistance had developed to *B. thuringiensis* H-14, the Madurai laboratory colony, Fort Kochi, and Gandhinagar (F_{18}) strains were bioassayed using *B.t.i.* (IPS-

Table 2. LC_{50} and LC_{90} values of *Culex quinquefasciatus* from unsprayed areas and areas sprayed with *Bacillus sphaericus* 2362 (SPH-88).

Area	LC_{50} (mg/liter)	95% fiducial limits Upper	95% fiducial limits Lower	LC_{90} (mg/liter)	95% fiducial limits Upper	95% fiducial limits Lower	Slope
1. Madurai (Lab)	0.004a[1]	0.006	0.002	0.024a	0.059	0.037	1.61a
2. Kallur	0.008a	0.013	0.006	0.028a	0.049	0.015	2.46b
3. Fort Kochi	0.008a	0.012	0.006	0.031a	0.055	0.018	2.19a
4. Mattancherry	0.012a	0.020	0.007	0.043a	0.101	0.018	2.26a
5. Indiranagar	0.015a	0.018	0.013	0.085a	0.114	0.063	1.74a
6. Vytilla	0.070a	0.084	0.058	0.600a	0.924	0.392	1.37c
7. Gandhinagar (F_{18})	49.78b	64.04	38.69	971.07b	1,877.63	502.22	1.00c

[1] Values followed by a common letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

82). All 3 were susceptible, with the larvae from Gandhinagar (F_{18} generation) showing the lowest LC_{50} (0.001 mg/liter) and LC_{90} (0.01 mg/liter) values, followed by the Madurai strain (0.007 and 0.02 mg/liter), and the Fort Kochi strain (0.01 and 0.04 mg/liter).

DISCUSSION

When it was first suspected that resistance to *B. sphaericus* had developed in the Kochi field population of *Cx. quinquefasciatus,* only the formulation of *B. sphaericus* 1593M that had been sprayed in the area was available for carrying out bioassays. Using this, an approximately 150-fold increase at the LC_{50} level was detected in the population from the sprayed area in comparison with an unsprayed area in Kochi. None of the populations from unsprayed localities differed significantly from each other although one (Vytilla), which was very close to the sprayed area, was 7 times higher at the LC_{50} level than a representative strain well isolated from the sprayed area. Tests carried out with the formulation, which because of its viscous nature could not be homogenized satisfactorily, needed to be confirmed using the standard primary powder. Therefore, all tests were repeated using *B. sphaericus* 2362 (SPH-88) after a delay of several months. All the susceptible strains fell in the same order as they had with the formulation of *B. sphaericus* 1593M, except for one (Kallur), which had changed from most susceptible to 2nd position. Vytilla had a 9 times higher LC_{50} value than Fort Kochi.

The field-collected larvae from the sprayed area could obviously not be tested against the standard, because the resistant strain had already been maintained in the laboratory for 17 generations under moderate selection pressure. It was not originally intended to select for high resistance, but it was thought that the field resistance might be unstable and that low selection pressure might be required for maintenance. However, LC_{50} values rapidly increased and at the 7th generation in the laboratory, there was an increase of 1,594 times in resistance at the LC_{50} level. At the 18th generation, which was tested using the standard powder, the LC_{50} had increased 6,223 times in comparison with the susceptible Fort Kochi strain.

It might not have been possible to obtain fresh samples of the field-resistant strain by going back to the field, as spraying had been discontinued, and this was followed by heavy monsoon rains that could have washed out larval habitats. Because the Vytilla strain maintained its relative position with about the same order of magnitude (7 and 9 times greater LC_{50} values with *B. sphaericus* 1593M and 2362 [SPH-88], respectively) in comparison with other susceptible strains, it is reasonable to assume that the LC_{50} of the resistant strain in the field would have been about 150 times that of the susceptibles with the standard powder also.

The Vytilla strain appears to be heterogeneous, as the LC_{50} and LC_{90} values were about 7–9 and 12–20 times greater than those of the presumably homogenous susceptible strains. This could be due to variation between natural populations. Georghiou et al. (1990) observed a population of *Cx. quinquefasciatus* with an LC_{95} value about 6 times that of the mean LC_{95} value for all the populations surveyed, although there was no previous exposure to *B. sphaericus.* However, in the present study the possibility of resistant females migrating to Vytilla from the adjoining sprayed area cannot be excluded. It is also possible that there may have been run off of the formulation of *B. sphaericus* 1593M from the sprayed drains.

It was previously believed (Davidson 1992) that high levels of resistance to bacterial insecticides would not be encountered in field populations. Experience with *B. thuringiensis* H-14 generally supported this view. *Bacillus thuringiensis* var. *israelensis* has been in operational use for the control of mosquitoes for many years but no instance of development of field resistance has yet been reported. For example it has been used against floodwater mosquitoes in Germany for more than 10 years without the development of field resistance (Becker and Ludwig 1993). High selection pressures with *B.t.i.* (IPS-82) applied in the laboratory have so far led to the development of only low levels of resistance (Vasquez-Garcia 1983,[1] Goldman et al. 1986). However, the recognition of resistance to *B. sphaericus* in field populations of *Cx. quinquefasciatus* in Brazil (M. H. Silva-Filha et al., unpublished data) and in Kochi, India, demonstrates that greater caution will have to be exercised when using this agent. In Kochi a relatively high level of resistance was detected in the field within 2 years of application of *B. sphaericus* 1593M. Subsequently in the laboratory an increase of about 6,000 times in LC_{50} value was obtained by the 18th generation subjected to low to moderate selection pressure. Georghiou et al. (1992) obtained an increase of 100,000-fold at the LC_{50} level in only the 12th generation in a population subjected to a very high (94–98%) selection pressure.

The Kochi resistant strain did not show any

[1] Vasquez-Garcia, M. 1983. Investigations of the potentiality of resistance to *Bacillus thuringiensis* ser. H-14 in *Culex quinquefasciatus* through accelerated selection pressure in the laboratory. Ph.D. dissertation. University of California, Riverside.

cross resistance to *B. thuringienisis* var. *israelensis,* as also found by Georghiou et al. (1992) in the Bakersfield resistant strain. Similarly the Kochi strain, selected for resistance by *B. sphaericus* 1593M was also resistant to *B. sphaericus* 2362, which belongs to the same serotype (H5a5b).

It is not clear why such a high level of resistance (×150) should have appeared in a natural population, which though under selection pressure due to *B. sphaericus,* must have been subject to constant immigration of females from adjoining unsprayed areas. A high initial frequency of the gene(s) responsible for resistance may have given this particular population a high propensity for selection. In any case, it is clear that baseline data on susceptibility of natural populations of mosquitoes to *B. sphaericus* should be obtained and all field trials should be monitored so that loss of susceptibility can be detected at an early stage and timely changes in control strategies can be initiated.

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