EFFECTIVENESS OF *BACILLUS SPHAERICUS* VAR. *FUSIFORMIS* (SSII-1) AS A POTENTIAL MOSQUITO LARVAL CONTROL AGENT: THE ROLE OF VARIATIONS IN NATURAL MICROBIAL FLORA IN THE LARVAL ENVIRONMENT[1, 2]

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ABSTRACT. Using *Bacillus sphaericus* var. *fusiformis* (SSII-1) we have clearly demonstrated that variations in the aquatic microbial flora can influence the observed lethal activity of SSII-1 over a range of 10^6, thus suggesting that natural variation in microbial flora may determine the efficacy of larval control by this mosquito pathogen.

Kellen, et al. (1965) demonstrated that a strain of *Bacillus sphaericus* var. *fusiformis* (Neide) had measurable larvicidal activity against 10 species of mosquitoes. Lethal dosages (ED_{50}) ranged from 10^7–10^8 spores/ml of maintenance fluid. Subsequently, Singer (1973) isolated *B. sphaericus* from dead mosquitoes received by the World Health Organization International Reference Center. Several of these isolates showed marked lethality for larvae of *Culex pipiens* var. *quinquefasciatus*, representing an apparent 10^4 improvement in activity over that reported by Kellen et al. Goldberg, et al. (1974) demonstrated that one of these isolates of *B. sphaericus* var. *fusiformis* (SSII-1), while equally effective against *Cx. tarsalis*, demonstrated low activity against *Aedes dorsalis, Ae. taeniorhynchus, Ae. aegypti* and *Anopheles freeborni*. Recently Davidson et al. (1974) reported that a chloroform-killed culture of SSII-1, in which the viable count was reduced from (1-3) × 10^9 mixed bacilli and spores/ml to a residual count of (1–40) × 10^8 viable spores/ml; (i.e., to a count equal to the original spore content), possessed a larvicidal activity against *Cx. pipiens quinquefasciatus*[2] nominally equal to that reported for untreated living bacteria. Their data also suggested that a lethal dose was nominally equivalent to an ingested dose of ca. 3 × 10^5 toxic bacterial cells/larva.

In this report we describe an improved, standardized procedure which has permitted us to observe the development of individual mosquito larvae under reproducible conditions until adults emerge. With this procedure, we have measured the lethality of SSII-1 for *Cx. pipiens quinquefasciatus*[2] and for *Cx. pipiens* (complex) larvae, and we have demonstrated marked variation in killing efficiency associated with natural or chance microbial flora that may be present during the period of larval development. The significance of such microbial flora variation is discussed in respect to bioassay of potential mosquito microbial pathogens and their proposed use for mosquito control.

MATERIALS AND METHODS

TREATMENT OF EGG RAFTS AND OF NORMAL MICROBIAL FLORA. Individual egg rafts were placed in small sterile plastic petri dishes containing 10 ml of a sterile alfalfa infusion. The normal or chance microbial flora of the egg raft provides the

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[2] Editor's Note: It is the policy of *Mosquito News* to follow recommendations of the National Mosquito Identification Service relative to nomenclature. *Cx. pipiens* and *Cx. quinquefasciatus* are considered "good" species. In this paper the authors have used mosquitoes from 3 sources which they prefer to identify as laboratory populations of *Cx. pipiens pipiens, Cx. pipiens quinquefasciatus* and *Cx. pipiens* (complex).

seed inoculum of the infusion, thus initiating a microbial flora to which newly hatched larvae must adapt. Ca, 100 live larvae per egg raft were produced within 48 hrs.

A marked reduction in the microbial flora of egg rafts and of the resultant microbial flora present in the aquatic medium during larval hatching, was achieved by adding 35 μg/ml of Gentamycin® (Schering Corp., Port Reading, N.J.) to the infusion prior to introducing the egg raft. Newly hatched larvae were rinsed twice with sterile distilled water prior to transfer to a second medium containing a desired microbial flora. Gentamycin treatment did not produce sterilization of the egg raft flora, but did minimize any effect upon the 2nd test flora under study. Sterilization with hypochlorous ion, rather than Gentamycin, was subsequently shown to produce microbial sterility in egg rafts; this method will be described elsewhere.

LARVAL REARING AND TEST TRAYS. Our rearing and experimental trays are made of clear polystyrene; they contain 25 wells or compartments, each measuring 2.8 × 4.1 × 1.6 cm, with a volume of 15 ml (Champion Packages Co., Chicago, IL.)

Two or three larvae were transferred into each well. Four ml of alfalfa infusion rearing medium (sterile or containing an established microbial flora) were added to each well. Trays were subsequently maintained at 26°C.

A second tray was used, inverted as a lid of the inoculated tray, to form 25 closed air/water cells, thus minimizing evaporative loss during the holding period of 10–14 days or until adult emergence is complete. Clear pieces of plexiglass were used as bases and as top weights for stacked trays to facilitate penetration of light and observation of growth.

FEEDING TEST LARVAE. Until pupation was completed, larvae were fed daily with a liquid nutrient preparation derived from alfalfa plus Tetramin®.

Sixteen grams of fresh frozen alfalfa new growth, mixed into 1 liter of water, was autoclaved for 45 minutes, and a clear infusion was obtained by filtering the solution through a coarse glass wool filter. This liquid, dispensed in quantities suitable for later feedings, was reautoclaved for 20 min at 20 pounds per in^2 to ensure sterility.

Four grams of Tetramin were suspended in 100 ml of water, allowed to become thoroughly wet, autoclaved for 20 minutes at 20 lb/in^2 and then stored until required, at 4° C. The Tetramin and alfalfa infusions were combined in a ratio of 1:99 (v/v) to serve as the initial hatching fluid, and 1:9 (v/v) for the maintenance fluid added to each well in 0.5 ml daily amounts. Nutrient fluids were warmed to the larval holding temperature of 26° C and shaken intermittently during use.

As the result of successive feedings until pupation (6–8 days), well-fluid volume increased from 4 ml to approximately 8 ml (i.e., ca 1/2 of the capacity of an individual well). Wells remained sufficiently clear to allow larval counts at all stages of mosquito development.

PREPARATION OF SSII-1 SUSPENSIONS. Seed cultures were incubated for 24 hr at 30° C on brain-heart infusion agar plates and held until used—or for a period of up to 1 month.

Test suspensions were grown in a proprietary medium, N2X (Nutrilite Products Inc., Buena Park, CA) developed for the large scale production of *B. thuringiensis*. Spore suspensions of SSII-1 were obtained from surface growth in petri dishes containing N2X plus 1.5% agar, incubated at 30° C for 24 hrs and harvested by scraping organisms into 10 ml of water. The resultant suspension, after heat-shocking for 20 min at 65° C, contained ca, 10^9 spores/ml.

Vegetative organism suspensions were obtained from 0.05 ml seed suspension inoculated into 50 ml N2X broth incubated in a 250 ml Ehrlenmeyer flask on a rotary shaker for 16 hrs at 30° C. Such suspensions produced greater than 99% vegetative organisms with yields of ca. 10^9/ml.

BACTERIAL CHALLENGE OF LARVAE. An 18 gauge blunted hypodermic needle was

employed to deliver 2 drops (0.04 ml) of a diluted bacterial suspension into each larval well containing 4 ml rearing fluid.

The criterion of "death" was failure of larvae or pupae to develop successfully into adult mosquitoes. Aqueous *B. sphaericus* (SSII-1) vegetative and spore counts and ingested organism counts normally were made after 2 days, and again after 8 days observation.

Cx. pipiens (COMPLEX) AND *Cx. pipiens quinquefasciatus* LARVAE: EXPERIMENTAL SOURCES. In earlier screening the authors noted no significant differences in response of larvae of *Cx. pipiens pipiens* and *Cx. pipiens quinquefasciatus* to challenge with *Bacillus sphaericus* var *fusiformis* (SSII-1). Both larval sources were colonies derived from previous field isolations in California. As a consequence, subsequent work in California was limited to *Cx. pipiens quinquefasciatus* (see data reported in summary form in Fig. 1).

Figs 2a and 2b summarize work done in Israel using a colony of *Cx. pipiens* (complex) isolated and identified by Dr. Joel Margalit. This colony was derived from larvae collected from a sewage outflow from a Kibbutz in the Golan Heights. Such sewage outflows are usually dominated by *Cx. pipiens* (complex) due to the high organic waste content of the water.

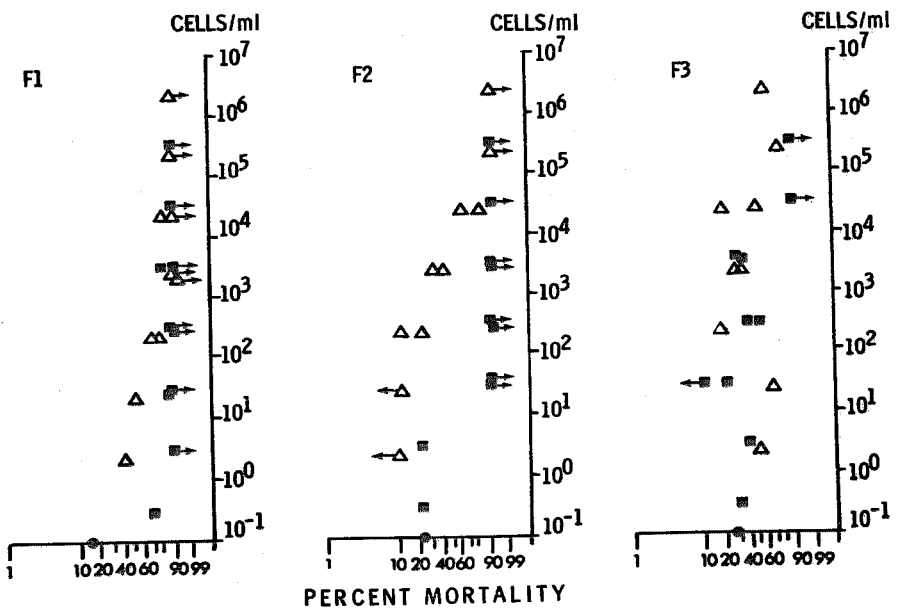

Fig. 1. Lethality of *Bacillus sphaericus* strain SS 11–1 for larvae of *Culex* (c) *pipiens quinquefasciatus* as a function of initial aqueous challenge, presence of different mixed natural microbial flora (F1, F2, F3), and of vegetative (■) or spore (△) phase of the challenge organism. (●) Control.

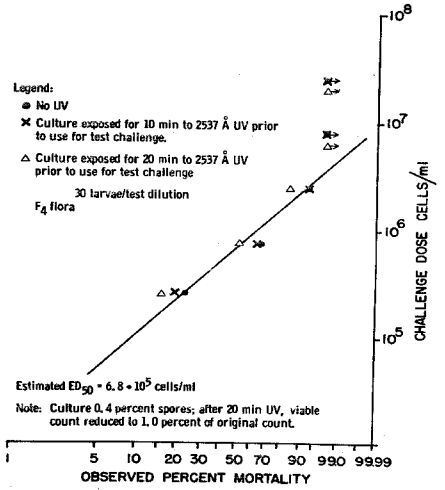

Fig. 2a. Comparative dose response of 1st–2nd instar *Culex pipiens* (complex) larvae to challenge with a 12-hr, 30 C N_{2x} agar test culture of *Bacillus sphaericus* (SS11–1) culture exposed for 0, 10 and 20 min to 2537 Å ultraviolet prior to use test challenge.

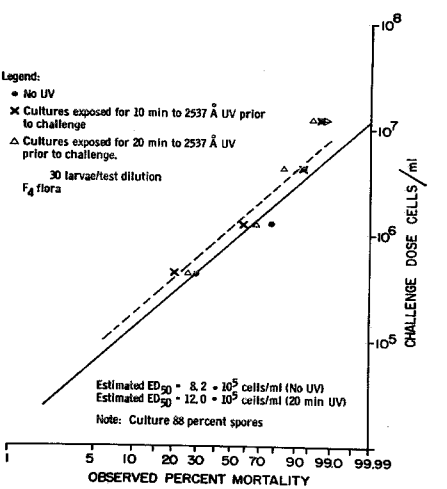

Fig. 2b. Comparative dose response of 1st–2nd instar *Culex pipiens* (complex) larvae to challenge with a 36-hr, 30 C N_{2x} agar test culture of *Bacillus sphaericus* (SS11–1) culture exposed for 0, 10 and 20 min to 2537 Å ultraviolet prior to use test challenge.

EXPERIMENTAL RESULTS

PRELIMINARY EXPERIMENTS. Individual egg rafts were allowed to hatch in alfalfa infusion to produce a variety of natural, or chance, microbial floras. Subsequent tests were made to determine the capacity of *B. sphaericus* (SSII-1) inocula, in either the spore or vegetative phase, to multiply and to kill larvae in each alfalfa infusion/microbial flora (AIMF) mix. With several of such floras, the ED_{50} of SSII-1 for larvae, in terms of the initially introduced spores and vegetative cells, ranged from nominally 1 to 10,000 organisms; however, it was noteworthy that regardless of initial numbers of spores or vegetative cells, dead larvae (3rd to 4th instar) contained $10^{5\text{-}6}$ *B. sphaericus* cells per larva.

EVIDENCE FOR REPRODUCIBILITY OF MICROBIAL FLORA (AIMF). Following these preliminary experiments, 3 laboratory floras were selected on the basis of low, intermediate, and high inhibition of *B. sphaericus* (SSII-1) lethality for larvae; these were designated F1, F2 and F3, respectively. In addition to these 3 laboratory "floras," a 4th flora (F4) was tested. This was a 1st passage flora obtained using an inoculum from a sewage pond (Kibbutz Hulda, Israel). The liquid in this sewage pond consisted primarily of the washing and environmental cleaning water of dairy cows.

Larvae in a selected AIMF were placed 2 per well, 50 per tray, 5 trays per flora, and were left to equilibrate for 24 hr. On the 2nd day (i.e., at approximately the 2nd larval instar), 2 trays were utilized for a spore titration, 2 trays for a vegetative titration using *B. sphaericus* (SSII-1), and 1 tray was retained as a test control. Larvae were observed daily during the larval, pupal and adult stages. Results are summarized graphically in Fig. 1, with each datum point representing observations on

10 larvae. It is evident that both the vegetative and spore test challenges with F1 present were extremely effective in killing test larvae; initial *B. sphaericus* spore numbers as low as 3000 cells/ml resulted in excellent control; vegetative organism challenge, however, provided excellent larval control with initial levels as low as 3 cells/ml.

The data obtained using F2 as the initial dominant larval flora showed an intermediate level of control with *B. sphaericus* at 30 vegetative cells/ml, whereas the spore phase was equally effective only at 30,000 spores/ml.

With F3 present as the initial microbial flora, 30,000 *B. sphaericus* vegetative cells/ml were required to achieve control conparable to that observed with 3 cells/ml in the presence of F1 flora. No satisfactory larval control was obtained, even at a challenge of greater than 10^6 spores/ml.

Viable bacterial assay of the larval test well, several days after test challenge, confirmed that the increased control levels observed in F1 were associated with enhanced aquatic replication of *B. sphaericus*. Additionally, no significant difference in viable microbial count per dead larva was observed as a function of initial challenge dose; in all instances, terminal viable counts of *B. sphaericus* in the aquatic medium, or in dead larvae, approximated 10^5–10^6 cells/ml or per larva, respectively.

The measured toxicity of SSII-1, was least when the larvae were challenged while being maintained in flora F4 (sewage pond flora). The estimated ED_{50} was $6.8 \cdot 10^5$ cells/ml (culture was 99.5% vegetative growth) (Fig. 2a). Aliquots of the test culture were exposed to ultraviolet (2537 Å) for 10 min and 20 min prior to use as test challenge, to further clarify the role of aquatic viable replication vs. cell toxicity. Following 10 min U.V. treatment, the viable count was reduced to 0.5% of its original value, a number equivalent to the initial spore count. A U.V. exposure of 20 min had no significant additional effect on the residual spore count. From the data graphically summarized in Fig 2, the estimated ED_{50} values from 0, 10 and 20 min U.V. cell treatment can be combined as a single mean value of $6.8 \cdot 10^5$ cells/ml, thus strongly suggesting that under these "worst test conditions," the role of SSII-1 against *C. pipiens* (*q*) is purely toxic rather than infective in nature.

Figure 2b summarizes the results of a comparable series of test challenges as was illustrated in Fig 2a, but using a test culture of SSII-1 which was incubated so as to result in a viable content of 88% spores (36 hrs incubation). The estimated ED_{50} dose was $8.2–12.0 \times 10^5$ cells/ml thus suggesting an increase in U.V. sensitivity and a slight decrease in toxicity per cell as spore formation progresses. Following heat shock of a test culture of *B. sphaericus* (SSII-1) for 20 min at 60°C the viable spore count was the same but no useful toxicity remained. Additionally, the incubation of *B. sphaericus* (SSII-1) on nutrient agar (Difco) markedly reduces cell toxicity for *Cx. pipiens* (complex) larvae.

DISCUSSION

The work of Davidson et al. (1975) clearly demonstrated that ingested, chloroform-killed vegetative cells of *B. sphaericus* can produce larval death with an efficacy comparable to that resulting from the same number of living cells. We have now shown that the infectivity of *B. sphaericus* (SSII-1) plays a minor role in defining a "lethal" dose, even though larval mortality is uniformly accompanied by a $10^{5\text{-}6}$ viable cells per larvae following challenge with viable organisms. The latter is further substantiated by the fact that heat-shocked *B. sphaericus* spores that are unable to multiply competitively in a selected larval aquatic environment (F4) are also unable to replicate significantly in larvae following ingestion. We suggest that results of Kellen et al (1965) may well have been biased by the chance microbial flora and nutrient growth media which he used since Singer (1973) was able to demonstrate a marked improvement in activity with a strain isolated by Kellen, but

tested under the same conditions used with his current isolate SSII-1. The range in response demonstrated with spores of SSII-1 in our current study can encompass the difference between the results of Singer (1973) and of Kellen et al. (1965).

Although spores of *B. sphaericus* can initiate growth in the presence of selected microbial floras, the fact that there are some natural floras; i.e., F4 (sewage pond flora), that inhibit replication, dictates that before *B. sphaericus* (SSII-1) can be reliably applied as a mosquito larval field control measure, the problem of "flora" inhibition should be solved or that a sufficiently high control dosage be utilized; i.e., based on $ED_{50} = \pm 7 \times 10^5$ cells/ml or ED_{95} of 3×10^6 cells/ml.

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PROTEIN BAND STUDIES OF THE SUBSPECIES IN THE *AEDES ATROPALPUS* GROUP[1]

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ABSTRACT. A study of the 4 subspecies in the *Aedes atropalpus* group was conducted using acrylamide gel electrophoresis to separate soluble proteins. Four anodically migrating protein bands were observed in whole-body homogenates of each subspecies. Differences in rates of migration and staining intensities of the protein bands were sufficient to differentiate the subspecies. Therefore, the biochemical data tend to support the 4 subspecies rather than the 2 species concept of the group.

Despite the recent attention given to the taxonomy of the *Aedes atropalpus* group its status still is not clear. O'Meara and Craig (1970) proposed 4 subspecies based on distinctive features in morphology, physiology, and behavior. All 4 subspecies were interfertile when crossed in the laboratory.

Zavortink (1972) made an extensive study of geographical distribution and morphological features and concluded that 2 distinct species were involved rather than 4 subspecies. The type-form *Ae. a. atropalpus* (Coquillett) was designated as *Ae. atropalpus* (Coquillett). Because no reliable distinguishing morphological characteristics could be found, the 3 remaining subspecies were synonomized under a reinstated species, *Ae. epactius* Dyar & Knab.

Recently, Brust (1974) presented data supporting the 2 species concept of the group. From matings in the laboratory it was shown that *Ae. a. epactius, Ae. a. perichares,* and *Ae. a. nielseni* crosses had a high genetic affinity, while *Ae. a. atropalpus* had a low genetic affinity when crossed with the other 3. Scanning electron micrographs of the chorionic sculpturing of the eggs revealed that *atropalpus* was distinct,

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