ISOLATION OF MOSQUITO-TOXIC BACTERIA FROM MOSQUITO-BREEDING SITES IN KENYA

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ABSTRACT. A large number of source materials were collected for isolating entomopathogenic bacteria from larval mosquito habitats in Kirinyaga District, Kenya. Mosquito-toxic bacteria were included among the numerous types of microorganisms isolated from the habitats. The pathogenic isolates shared common structural characteristics; they were gram-positive, spore-forming bacilli that produced parasporal inclusions conferring broad-spectrum larvicidal activity against *Anopheles*, *Culex* and *Aedes* mosquitoes. Based on structural and growth characteristics, coupled with larvicidal activity, the pathogenic isolates were tentatively identified as variants of *Bacillus thuringiensis*. Although the collection consisted of a variety of items including soil, silt and mud, the most productive materials were larval bodies. Using healthy mosquito larvae held in a fully permeable plastic bottle, a baiting technique was developed as a means of recovering bacteria from the environment.

Following the isolation and identification of *Bacillus thuringiensis* subsp. *israelensis* by Goldberg and Margalit (1977) and de Barjac (1978), respectively, numerous attempts have been made to isolate and develop other strains that have shown varying degrees of pathogenicity to mosquito larvae. Padua et al. (1980) identified *B. thuringiensis* serotype 10 with a preferential toxicity to mosquito larvae. Another strain of *B. thuringiensis* (serotype 8a: 8b), found selectively toxic against mosquito larvae, was isolated by Padua et al. (1984). During a screening program in Israel, Brownbridge and Margalit (1986) isolated several *B. thuringiensis* strains toxic to mosquitoes. As reviewed by Davidson (1982), *B. thuringiensis* subsp. *israelensis* is lethal to all filter-feeding species of mosquito larvae and is highly toxic to blackfly larvae. Besides *B. thuringiensis* subsp. *israelensis*, another mosquito-toxic bacterium is *Bacillus sphaericus* Neide (Davidson et al. 1975, Kellen et al. 1965, Singer 1974).

Although a wide variety of mosquito-active bacteria have been isolated in several geographical areas, isolation of additional entomopathogens is required due to the economic cost and restrictions imposed on the use of foreign microorganisms. Thus, the present study was initiated primarily to isolate and screen entomopathogenic bacteria from the Kenya environment, with the hope of finding some local pathogens as an alternative to the use of imported agents. In addition, there is the potential for isolation of new pathogens or serotypes of previously known pathogens possessing characteristics that make them more suitable for development and field application.

Source materials for isolation (mosquito larvae, silt, soil, mud) were collected in sterile bags from mosquito breeding habitats identified at Mwea Rice Irrigation Settlement and Sagana Fish Culture Farm, both areas situated in Kirinyaga District, Kenya. The bulk of the materials collected consisted of soil and mud since dead mosquito larvae initially proved difficult to find. By means of a plastic bottle (Fig. 1), a technique was developed to overcome the problem of obtaining dead larvae. A screw-top rectangular plastic bottle (10 × 3.5 × 3 cm) was perforated to allow adequate air circulation and entry of water but prevent exit of mosquito larvae introduced into the bottle. The perforated column was 4 cm high and occupied about the top half, on all sides of the bottle. The bottom end (4 cm high) of the bottle, not perforated, was filled with water to accommodate 40 disease-free, laboratory reared third instar *Aedes aegypti* (Linn.). Shortly after introducing the larvae into the

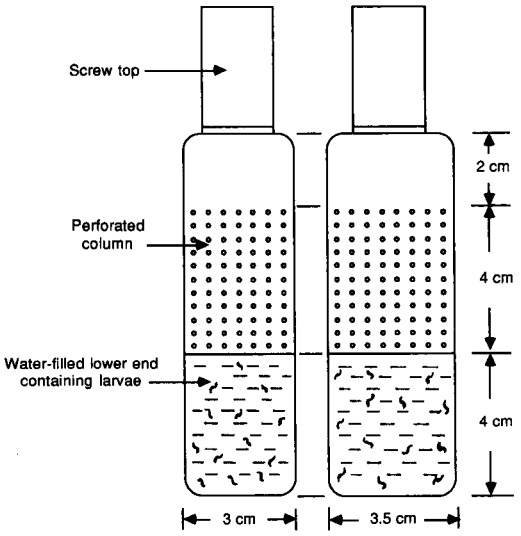

Fig. 1. Diagram of the "baiting" apparatus.

bottle, the top was replaced, and the bottle allowed to float on the surface of the breeding habitat for 72 h to facilitate exposure to possible bacterial infection. At the end of the exposure period, the bottle was collected and taken to the laboratory where the larvae were transferred into a sterile petri dish and kept at 4°C until required for isolation. Five habitats were sampled using this baiting technique.

In the laboratory, the source materials were placed in small, loosely covered containers and held at room temperature until they were thoroughly dried. The larval materials were first washed with 70% ethyl alcohol and sterile distilled water to wash away surface bacteria. Under aseptic conditions, each sample of source material was homogenized and dispensed into a conical flask containing 25 ml sterile phosphate buffered saline previously adjusted to pH 7.2. The contents of the flask was agitated vigorously with a vortex mixer prior to heating at 78°C for 15 min in a waterbath. A sample (0.1 ml) of the heated suspension was pipetted aseptically onto the surface of a sterile solid medium in a plate containing 5 g yeast extract, 10 g tryptone, 5 g NaCl, 20 g agar per liter of distilled water (Miller 1972). The suspension was spread evenly over the agar surface using an L-rod and the plate incubated in the oven at 30°C. After 24 h incubation, the plate was examined for bacterial growth and the representative colonies were subcultured and kept at 4°C until required for toxicity testing.

A loopful of bacterial growth was used to inoculate 50 ml PGSM medium (Brownbridge and Margalit 1986) placed in 250 ml Erlenmeyer flask. The medium had been previously adjusted to pH 7.2 with NaOH and autoclaved at 121°C for 20 min. The flask was incubated on a rotary shaker maintained at 180 rpm and 28°C for 48 h. Toxicity tests were carried out using early fourth instar larvae of laboratory-reared *Ae. aegypti* mosquitoes. The tests were made in 100 ml glass beakers each containing 45 ml dechlorinated water and 20 larvae. After 48 h incubation, 5 ml aliquots from the PGSM culture were added to the contents of the beaker and larval mortality recorded after 24 h. Isolates causing more than 80% mortality were retained for further evaluation using powders prepared by the lactose coprecipitation method (Dulmage et al. 1970), utilizing 72 h PGSM cultures.

Bioassays followed the method described by Abdel-Hameed et al. (unpublished data). Twenty-five milligrams of the powder was homogenized in 10 ml of sterile distilled water and the resulting suspension serially diluted over a range from 25×10^{-2} to 25×10^{-7}. The toxicity of each concentration was assayed with 20 larvae per beaker, using three species of mosquitoes (*Anopheles arabiensis* Patton, *Culex quinquefasciatus* Say, *Ae. aegypti*). Three replicate assays and a control were performed in each case, and mortality was recorded after 24 h exposure. Mortality data were transformed to percentages, subjected to probit analysis, and regression equations calculated (Finney 1971). Only data points between the lowest concentration that caused 100% mortality, and the highest that caused 0% were used in the analysis for each mosquito species/isolate combination. Confidence limits at the 95% probability were calculated for the LC_{50} of each species/isolate combination (Sokal and Rohlf 1981).

Three hundred samples of source materials comprising soil, 120 (40%); mud, 90 (30%); silt, 55 (18%); fish feces, 30 (10%); and mosquito larvae, 5 (2%) were collected from numerous mosquito habits. Forty-two of the samples produced several types of bacteria but only 5 of these contained mosquito-toxic bacteria. The larval samples (all of which were used in the baiting technique) constituted about 2% of the total source materials but accounted for 3 (60%) of the mosquito-active isolates. Each of the silt and fish feces components represented 20%, while both the soil and the mud sources contained no pathogens. The LC_{50} in the various mosquito species/isolate combinations are presented in Table 1. Probit analysis regressions were significant at $P = 0.001$ in all cases and r^2 values were generally high. However, because of the few data points used in the analysis, the 95% confidence limits of LC_{50} were overlapping between almost all of the species/isolate combinations, indicating no significant differences.

In comparison with the other materials, larval bodies were potentially more pathogen-concentrated and therefore were productive source materials for isolating entomopathogenic bacteria. Demonstrating a success rate of 60%, the baiting technique developed in this study proved an efficient means of recovering bacteria from the environment. The method has the advantage of directly exposing the larval material to an extensive cover of habitats, thereby increasing the chances of picking up infection and concentrating the pathogens. Depending on the size, one baiting bottle could hold as many as 200 larvae. Several of these bottles could be used to facilitate sampling large expanses of breeding habitats such as rice fields, fish ponds and marshy biotopes. The approach could also allow for the use of different species of mosquitoes for baiting purposes in order to enhance the possibility of recovering a wider range of host-specific pathogenic material.

Table 1. LC_{50s} and regression slopes of isolates/mosquito species combinations.

Isolate	Mosquito species	Regression slope ± SE	LC_{50} with 95% confidence limits mg/liter
B42 (silt)	*Anopheles arabiensis*	2.41 ± 0.10	0.1 (0.04–0.24)
	Aedes aegypti	2.02 ± 0.57	0.4 (0.0–21.26)
	Culex quinquefasciatus	2.34 ± 0.43	0.3 (0.0–23.3)
B51 (fish feces)	*An. arabiensis*	2.34 ± 0.21	0.4 (0.02–0.8)
	Ae. aegypti	2.16 ± 0.36	0.2 (0.0–7.3)
	Cx. quinquefasciatus	3.72 ± 0.26	0.3 (0.1–0.9)
B53 (larvae)	*An. arabiensis*	2.42 ± 0.06	0.08 (0.05–0.13)
	Ae. aegypti	2.33 ± 0.44	0.3 (0.0–27.8)
	Cx. quinquefasciatus	3.72 ± 0.34	0.03 (0.0–0.13)
B54 (larvae)	*An. arabiensis*	2.32 ± 0.18	0.1 (0.02–0.6)
	Ae. aegypti	1.70 ± 0.42	0.1 (0.0–55.3)
	Cx. quinquefasciatus	3.72 ± 0.28	0.3 (0.1–0.97)
B55 (larvae)	*An. arabiensis*	2.33 ± 0.18	0.12 (0.02–0.6)
	Ae. aegypti	3.72 ± 0.56	0.4 (0.0–4.2)
	Cx. quinquefasciatus	3.72 ± 0.31	0.3 (0.1–1.1)

The identity of the isolates has not yet been confirmed, although taxonomic evidence suggests that they are probably *B. thuringiensis*. Further studies are being carried out. These studies, which include characterization and comparative toxicity testing, will highlight the taxonomic identity as well as potential characteristics of the isolates that have demonstrated broad-spectrum larvicidal activity against *Anopheles*, *Culex* and *Aedes* mosquitoes.

We are grateful to Thomas R. Odhiambo, Director of the ICIPE, for his support and encouragement. We also thank William A. Overholt and David Munyinyi for their assistance in analyzing the data. The technical assistance of M. Miti is also greatly appreciated.

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