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Novel cost-effective method of screening soils for the presence of mosquito-pathogenic bacilli

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Aims: The aim was to simplify the cumbersome conventional process of isolating virulent bacilli, which involves isolating all bacilli strains from a source followed by screening for strains that are effective for bio-control of mosquito vectors.

Methods: A new simplified technique involving eight steps was devised for screening soil samples for the presence of mosquito-pathogenic bacilli before isolating individual strains.

Results: Using the new technique, we obtained eight bacilli strains (KSD1-8) showing pathogenic activity against mosquito larvae from three out of 10 soil samples screened. These strains were characterized, identified and the main bioassay tests were performed with three most promising strains (KSD-4, KSD-7 and KSD-8), and their pathogenic activity against *Anopheles stephensi* Liston, *Culex quinquefasciatus*, Say and *Aedes aegypti* Linnaeus compared well with commercial reference strains of *B. thuringiensis israelensis* and *B. sphaericus*.

Significance and Impact of the Study: The new technique of screening soil samples for the presence of virulent pathogenic strains of bacilli against mosquito larvae proved quick, efficient and cost effective.

INTRODUCTION

Bacillus-based bio-toxins are gaining importance in the control of insecticide-resistant populations of insect pests and vectors of human diseases (Mulla 1991; Priest 1992; Peferon 1997). A number of formulations of these effective strains are now commercially available for large-scale use (de Barjac 1990; Mulla 1991). Endotoxins produced by *Bacillus* species are often species-specific and, unlike chemical insecticides, do not contaminate the environment, as the toxins have lesser residual efficacy and are generally safe for non-target organisms (Lacey *et al*. 2001; Siegel 2001). Consequently, there is a need to search for and isolate new indigenous and more effective strains.

Members of the genus *Bacillus* are ubiquitous organisms and can be isolated from various habitats including soil, water and dead/dying insects (Goldberg and Margalit 1977; Menon *et al*. 1982; Manonmani *et al*. 1987; Travers *et al*. 1987; Gupta *et al*. 1991; Hastowa *et al*. 1992; Orduz *et al*. 1992). Martin and Travers (1989) reported the isolation of *B. thuringiensis* in 785 out of 1185 soil samples, which shows that in 70% of the samples, *B. thuringiensis* alone was present. On the other hand, Manonmani *et al*. (1990) found *B. sphaericus* in only nine (3·37%) of 139 soil samples screened for the *Bacillus* species.

The current method of isolating *Bacillus* strains pathogenic to the mosquito is cumbersome and time-consuming, as it involves plating of a large number of soil samples, and selection, purification and testing for the pathogenicity. Thus there is considerable wastage of media, time and effort before one hits upon a promising strain. To address this problem, we devised a soil screening method that could reveal the presence of mosquito-pathogenic bacilli in soil samples before their isolation. The activity of pathogenic bacilli strains isolated using the new technique was evaluated against the larvae of three important mosquito vector species.

MATERIALS AND METHODS

The upper crust of soil in mosquito-breeding habitats was chosen as a source of pathogenic bacilli, as moribund larvae were difficult to obtain from the field. This choice was

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influenced by the fact that *Bacillus* spores are known to settle rapidly in bodies of water (Mulligan *et al*. 1980; Ignoffo *et al*. 1981; Silapanuntakul *et al*. 1983; Davidson *et al*. 1984; Orduz *et al*. 1992). Soil samples were collected aseptically from the bottom of only those stagnant ponds and paddy fields where the immature stages of mosquito were prevalent during the post monsoon months.

The protocol developed for screening soils for the presence of *Bacillus* strains for mosquito was as follows.

Step 1: aseptic collection of soil.

Step 2: addition of 10 ml of Luria Bertani broth (Sambrook *et al*. 1989) to 0.5 g of soil in a 125-ml Erlenmeyer flask.

Step 3: incubation with shaking (200 r.p.m.) for 4 h at 30 °C.

Step 4: 1-ml aliquot removed and heat shocked at 65 °C for 10 min in a prewarmed 5-ml glass tube.

Step 5: 0·1 ml of the sample removed and added to 1 ml of L-medium and incubated for 24 h at 30 °C.

Step 6: 1 ml of NYSM (sporulating medium) (Myers and Yousten 1978) added and incubated for 2 d at 30 °C.

Step 7: 0·1 ml of culture was taken for preliminary toxicity testing against mosquitoes (10 mosquito larvae in 10 ml of sterile distilled water).

During the protocol, *Bacillus* strains were selected by acetate (step 2), heat treated (step 4) and grown on L-medium (Step 5). Mg++, Mn++ and Ca++ ions in NYSM help support bacterial sporulation (step 6). These sporulating cultures were then used for preliminary toxicity testing against Anopheles/Culex larvae (step 7). In this step the mortality in III instar larvae was recorded after 24 and 48 h and concurrently positive and negative controls were maintained for comparison. The III instar larvae of mosquito species *Anopheles stephensi* and *Culex quinquefasciatus* used in bio-assays were obtained from laboratory reared colonies at the Malaria Research Centre (Indian Council of Medical Research) at Goa, India. The corrected mortalities were determined using Abbot's formula (Mittal *et al*. 1993). Experiments were rejected if the mortality was more than 20% in the negative control, which contained a similar number of larvae in water as in the experiment.

$$\%\ \text{Mortality} = \frac{(\%\ \text{mortality in the experiment}) - (\%\ \text{mortality in the control})}{100 - (\%\ \text{mortality in the control})} \times 100$$

All eight isolates (KSD1-8) were identified to species level using the taxonomic keys of Gordon (1973) and Berkeley (1984). Electron micrographs of the three most promising virulent strains (KSD-4, 7 and 8) and two reference strains, *B. thuringiensis israelensis* 164 (H-14) and *B. sphaericus* 161 (H5a5b), were prepared. The main bio-assays of these three strains against III instar larvae of *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti* Linnaeus were performed following the standard WHO procedure (WHO 1985), and their LC_{50} values were determined with MS log-probit software (POLO, Le Ora Inc., Berkeley, CA, USA).

RESULTS

Identification of sites containing mosquito-pathogenic bacilli

Using the developed protocol, soil samples from 10 mosquito breeding habitats in Goa state located on the western coast of India were screened. Samples I, II and VI produced a mortality against the III instar larvae of either *Anopheles stephensi* Liston or *Culex quinquefasciatus* Say of more than 50% (Fig. 1).

Isolation of *Bacillus* strains that were pathogenic against mosquitoes

The three soil samples containing virulent putative strains were further processed to isolate strains of *Bacillus*, using acetate selection (Travers *et al*. 1987; Carozzi *et al*. 1991). From these soil samples, eight different *Bacillus* strains were isolated and identified as *B. pumilus* (KSD-1), *B. sphaericus* (KSD-2), *B. brevis* (KSD-3), *B. sphaericus* (KSD-4), *B. subtilis* (KSD-5), *B. stereothermophilus* (KSD-6), *Bacillus* sp. (KSD-7) and *B. sphaericus* (KSD-8). Two isolates, KSD-4 and KSD-7, were deposited in the National Microbial Culture Collection facility at the Institute of Microbial Technology (IMTECH) at Chandigarh, India, where they were re-identified as *Bacillus sphaericus* and *B.* sp. and were assigned MTCC (Microbial Type Culture Collection) numbers 3672 and 3673, respectively.

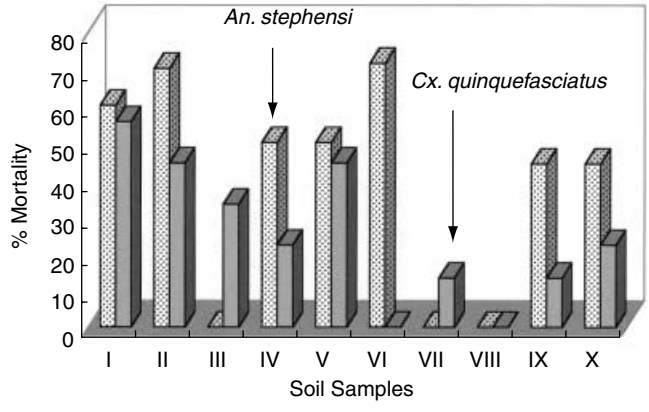

Fig. 1 Results of new techniques developed to screen soil samples for the presence of mosquito-pathogenic bacilli

Table 1 LC_{50} value of three isolates (KSD 4, 7 and 8) from Goa, India and from Russian commercial reference strains against III instar larvae of three mosquito vector species

Isolate	Mosquito species		
	Culex quinquefasciatus	*Anopheles stephensi*	*Aedes aegypti*
B. sphaericus KSD-4 (MTCC 3672)	0·1711 μg ml^{-1}	2·17 μg ml^{-1}	1·008 μg ml^{-1}
Bacillus sp. KSD-7 (MTCC 3673)	0·4506 μg ml^{-1}	3·06 μg ml^{-1}	0·9419 μg ml^{-1}
B. sphaericus KSD-8	0·0008 μg ml^{-1}	7·79 μg ml^{-1}	282 μg ml^{-1}
B. thuringiensis israelensis 164 (H-14)	0·92 μg ml^{-1}	11·97 μg ml^{-1}	4·09 μg ml^{-1}
B. sphaericus 161 (H5a5b)	0·138 μg ml^{-1}	1·75 μg ml^{-1}	2·92 μg ml^{-1}

The bioassay tests were conducted with isolates KSD-4, 7 and 8, as they showed virulence even after repeated subculturing against the larvae of three vectors: *Anopheles stephensi* (vector of urban malaria in India), *Culex quinquefasciatus* (filaria vector) and *Aedes aegypti* Linnaeus (dengue/DHF vector). The LC_{50} values of the three isolates KSD-4, 7 and 8 compared well with *B. thuringiensis israelensis* 164 (H-14) and *B. sphaericus* 161 (H5a 5b) strains obtained from Russian commercial powder formulations Bacticide and Sphericide, respectively (Table 1).

DISCUSSION

Travers *et al*. (1987) isolated strains of *Bacillus* by adding 0·5 g of soil to 10 ml of L-broth in a 125-ml triple baffled flask. The L-broth was buffered with 0·25 M sodium acetate. This mixture was shaken (250 r.p.m.) for 4 h at 30 °C. At the end of this time a sample was taken, heat treated at 75 °C for 1 h in a prewarmed 5-ml glass tube, plated on L-agar and grown overnight at 30 °C. Random colonies were picked on T3 medium and allowed to sporulate overnight at 30 °C. The cultures were checked for the presence of crystals, which was the criterion adopted to confirm isolates of *Bacillus thuringiensis*. In this method, most of the other spore-forming bacteria (except bacilli) and all non-spore-forming organisms in the soil/dirt samples were eliminated. The acetate inhibited the germination of desired bacteria, while the unwanted bacteria germinated. The latter, which were in a vegetative state, were then eliminated by a controlled heat treatment in the next step of heat shock.

Following this technique they isolated *Bacillus thuringiensis* from environmental samples with more than 10^9 bacilli g^{-1} of soil. Although this method has been successfully and extensively employed (Martin and Travers 1989; Carozzi *et al*. 1991; Kaelin *et al*. 1994) for the isolation of soil *Bacillus* species, each sample is required to undergo a lengthy isolation procedure and it results in the selection of both toxic and atoxic strains of spore-forming bacilli.

The proposed entomopathogenic screening procedure prescreens soil containing *Bacillus* strains before isolating them through the selection process. With this process three out of 10 soil samples were found to contain *Bacillus* strains, which were pathogenic to either Culicine or Anopheline larvae. The significance of this new technique lies in the identification of those soil samples that possessed highly virulent mosquito-pathogenic bacilli (three out of 10 soil samples in this case), separating them from those that were less effective during preliminary tests (Fig. 1). On the basis of their morphological and bio-chemical characteristics, the strains were identified as belonging to *Bacillus* spp. (Fig. 2). These cultures retained 100% viability when stored in 20% v/v glycerol at −20 °C. Thus the new technique can be effectively employed for screening of soils containing mosquito-pathogenic bacilli.

Chemical insecticides are the mainstay of mosquito control programmes in most parts of the world. When compared with entomopathogens, the primary consideration is efficacy and cost. However, the major advantages of the use of entomopathogens are their safety to non-target organisms, reduced pesticide residues in food, preserved natural enemies and resultant increased bio-diversity (Lacey *et al*. 2001). However, their utilization in vector control programmes, particularly in the integrated management of disease vectors, would depend upon better screening methods, the isolation of highly virulent strains against mosquitoes, better formulations and mass production at an affordable cost.

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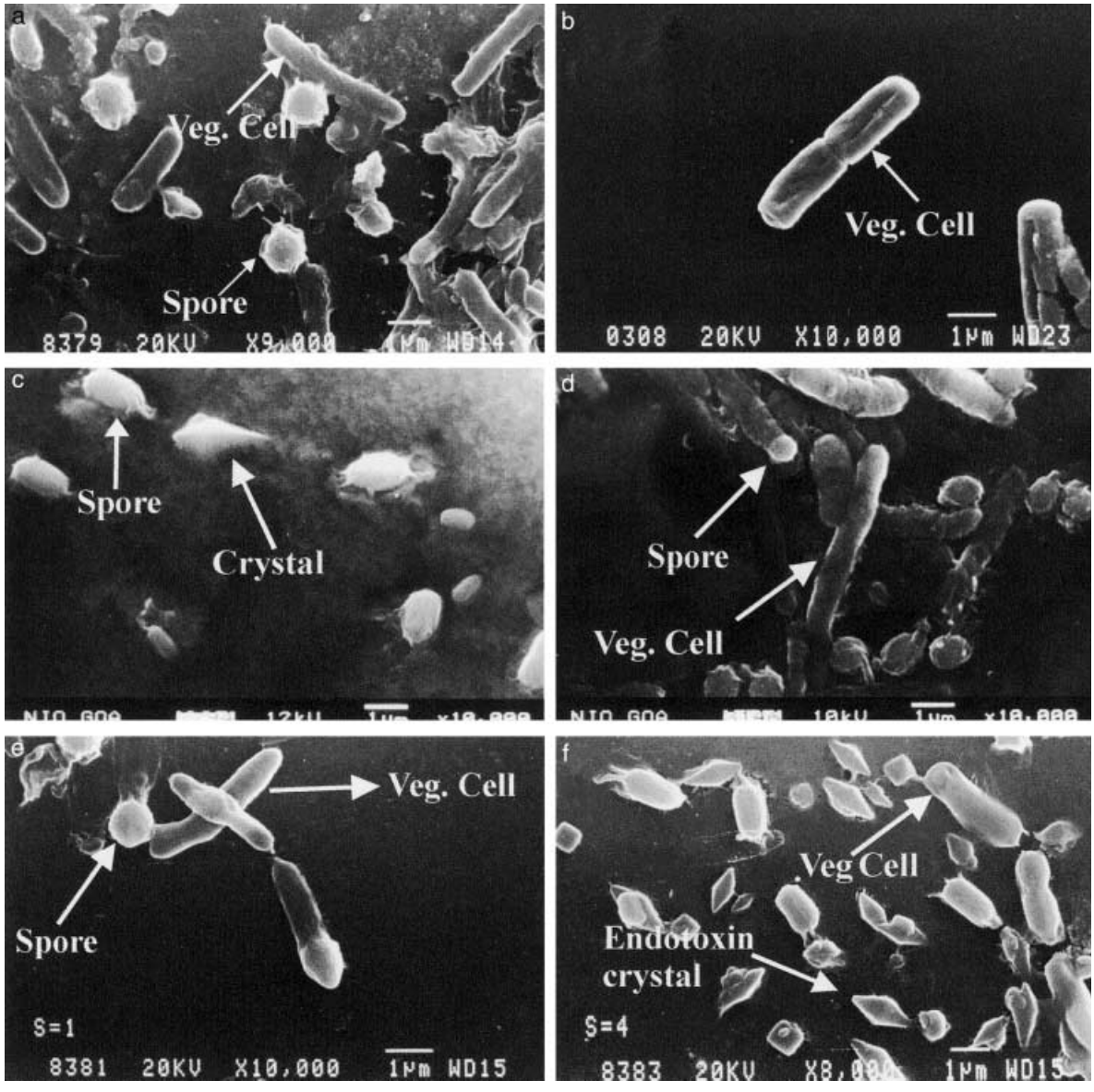

Fig. 2 Electron micrographs of mosquito-pathogenic bacilli isolated from soil samples from Goa, India. (a) *Bacillus pumilus* (KSD-4); (b,c) *B. Sp.* (KSD-7); (d) *B. Sphaericus* (KSD-8), and (e,f) reference strains; (e) *B. sphaericus* 101 (H5a5b) and (f) *B. thuringiensis israelensis* 164 (H-14) isolates from Sphaericide and Bacticide commercial formulations, respectively

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