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LABORATORY EVALUATION OF SIX MOSQUITOCIDAL *BACILLUS SPHAERICUS* STRAINS ISOLATED FROM AQUATIC SNAILS

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Abstracts

Six pathogenic *Bacillus sphaericus* strains have been isolated from the aquatic snails, *Melanoides tuberculata* and *Physa waterlotti* both non-hosts of schistosomes. The snails were collected from burrow pits in Accra, Ghana. Final whole culture preparations tested on four species of aquatic snails yielded no mortality. On the other hand, all the preparations proved very active against fourth instar *Culex quinquefasciatus* larvae. In all cases, the levels of larvicidal activity exhibited by the strains seemed to be similar with LC_{50} values (dilution) ranging from 2.6×10^{-6} to 8.0×10^{-6}. Values obtained in terms of cell counts ranged from 18×10^3 to 8.0×10^3 colony forming units per milliliter (C.F.U/ml), and in terms of spore counts the values were between 7.7×10^2 and 2.0×10^3. In all cases maximum larvicidal activity seemed to occur within 24 hours. The results of the investigation showed that insecticidal *B. sphaericus* strains could occur in organisms other than insects. Thus, the search for these strains should be widened to include other organisms occurring in insect breeding habitats.

Résumé

OFORI, J.: *Evaluation au laboratoire de six souches moustiquecidal de* Bacillus sphaericus *isolé d'escargots aquatiques.* Six souches pathogènes *Bacillus sphaericus ont été isolé* d'escargots aquatiques, *Melanoides tuberculata* et *Physa waterlotti* tous deux non-hôtes de schistosomes. Les escargots étaient ramassé de terrier sous terre à Accra du Ghana. Les préparations de culture complète finale testées sur quatre espèces d'escargots aquatiques ne provoquaient pas de mortalité. D'autre part, tous les préparations prouvaient très actives contre 4e stade de larve de *Culex quinquefasciatus.* Dans tous les cas les niveaux d'activité larvicidale montraient par les souches semblent être semblable avec les valeurs LC_{50} (dilution) variant entre 2.6×10^{-6} et 8.0×10^{-6}. Les valeurs obtenues sur le plan des comptes de cellule variaient entre 1.8×10^3 et 8.0×10^3 unité formant de colonie par millilitre (UFC/ml) et sur le plan des comptes de spore, les valeurs variaient entre 7.7×10^2 et 2.0×10^3. Dans tous les cas l'activité larvicidale maximum semblait d'avoir lieu en 24 heures. Les résultats de l'investigation montraient que les souches insecticidales *B. sphaericus* pourraient se produire dans les organismes outre que les insectes. Donc la recherche pour ces souches devrait être élargi de comprendre d'autre organisme se produisant dans les habitats de reproduction d'insecte.

Introduction

Many of the development projects undertaken in the tropics and particularly in Ghana have resulted in the creation of habitats which favour the breeding of vectors of water associated diseases. (Hunter, Luis & David 1983). Thus, virtually all the water conservations projects involving the construction of dams and impoundments, and some constructional projects involving the excavation and creation of burrow pits have created conditions which enhance the breeding of mosquitoes, water snails and simulium flies (blackflies). Thus, vector-borne disease such as malaria, filariasis, schistosomiasis and onchocercia-

sis have been on the increase (PEEM, 1987). Meanwhile, cyclops, vectors of dracunculiasis (guinea worm), flourish in areas of poor water supply.

One of the effective ways of combating these diseases is to control the vectors. Hitherto, efforts at controlling these vectors have relied largely on chemical pesticides. These chemicals have, however, occasioned certain side effects. These include the development of resistant strains among the vectors (Davidson *et al.*, 1977; Mulla *et al.,* 1984b; TDR, 1985), their long lasting and hazardous effect on both the environment and non-target organisms and their rising cost. This is, in fact, a global problem and research efforts in vector control is currently aimed at developing alternative non-chemical measures that would avoid the above unwelcome side-effects.

The most promising method to date seems to be biological control, i.e. using organisms which act as natural enemies of these vectors and which naturally regulate their population densities (TDR, 1985). Biological control agents in the form of pathogens, parasites, predators or competitors tend to be specific for the vectors they attack and they are, therefore, safe to non-target organisms including mammals (Davidson *et al.,* 1977; TDR, 1985). In addition they pose virtually no problem to the environment and resistance is not easily developed. In planning an approach to the use of biocontrol agents the important factors to be considered include production technology, safety, specificity, efficacy and cost (NAS, 1979). Other factors include storage and convenience of application (TDR, 1985). A good biological agent must satisfy all or most of these criteria. On the basis of the above, two spore-forming bacteria, *Bacillus thuringiensis* H-14 and *B. sphaericus,* have emerged as the most promising biocontrol agents. The larvicidal properties of these bacteria have been found to be due to insecticidal proteins produced by these bacteria (TDR, 1985).

B. thuringiensis H-14 was first isolated from Israel in 1976 from a soil sample from a mosquito breeding site. It has been found to be very active against a wide range of mosquito species (Goldberg & Margalit, 1977; de Barjac & Coz, 1979; Frommer *et al.,* 1980; Gaugler & Finney, 1982). In spite of its high level of activity, however, it has been found to be ineffective in polluted waters in which most mosquitoes breed (Lacey, Urbina & Heitman, 1984). In addition, it has little or no residual activity (Margalit *et al.,* 1985). This strain reached operational status about two decades ago and is now used in major vector control programmes including the Onchocerciasis control Programme in the Volta Basin in West Africa.

B. sphaericus strains have received much attention lately due to their ability to operate in polluted waters (Nicolas, Dossou-Tovo & Hougard, 1987; Mulla *et al.,* 1997; Yadav, Sharm & Upadhyay, 1997). They are also better able to persist in the environment and recycle in the cadaver of larvae (Mulla *et al.*, 1984a; Hertlein, Lacey & Miller, 1979) thus providing a replenishable source of larvicide.

Though these microbial insecticides are available for use in mosquito-control programmes, their high cost makes their large-scale application unfeasible in developing countries where hard currency resources are limited.

It was against this background that studies were initiated to search for and evaluate local potent strains of *B. sphaericus* and to develop them as mosquito larvicides for field application.

All the *B. sphaericus* strains known to date have been isolated from insects or from soil and habitats of insect larvae (Goldberg & Margelit, 1977, Wickremensinghe & Mendis, 1980; Weiser, 1984; Lysenko *et al.,* 1985). The present paper presents the results of laboratory evaluation of six pathogenic *B. sphaericus* strains isolated from aquatic snails. They were designated IAB 467, 471, 477, 481, 482.1 and 482.2 all of which belong to serotype H6.

Experimental

All the six pathogens were isolated from *Melanoides tuberculata* and *Physa* spp. These

snails are uaually found on lake shores, burrow pits, rice fields and drains, habitats which also breed mosquitoes. The snails used in this study were collected from burrow pits which had been left uncovered for a prolonged period. They were transported to the laboratory in sterile cups and were identified, washed and dried in an oven at 40 °C for 24 h. Each snail was crushed in a mortar and its shell aseptically removed. The flesh was macerated and suspended in 5 ml sterile deionised water. The suspension (stock) was pasteurized at 80 °C for 12 min and cooled in an ice bath. Dilutions of the suspension were inoculated into conical flasks containing MBS broth and 0.1g/ml of streptomycin sulphate. This selects for *B. sphaericus* strains (Kalfon *et al.*, 1983). The flasks were incubated on an orbital shaker with constant agitation at 240 rpm for 24 h. Samples were removed periodically, stained and examined microscopically for purity. After 24 h incubation 2 per cent of the inoculum was sub-cultured into similar flasks. A third flask containing 100 ml media was treated as above and incubated for 48 h.

The resultant suspension, the final whole culture (FWC) was used to prepare 10-fold dilution series in deionised water. These were made in plastic cups and assayed against 25 fourth instar *Cx. quinquesfasciatus* larvae. These larvae were obtained from egg rafts collected from an aquarium tank containing hay infusion. They were hatched in dechlorinated water and the larvae were fed with cerelac (a baby food) fortified with yeast extract. Mortality from each flask and the LC_{50} range were noted.

Further bioassay procedure

Five dilutions per suspension were prepared within the LC_{50} range such that at least three points were obtained between 16 and 84 per cent mortalities at 48 h exposure. One hundred larvae were tested per dilution and these were distributed into four cups. Controls were run alongside the test. Control mortalities were corrected by Abbot's formula (Abbot, 1925). The assay was repeated three times. The larvae were fed on the first day only. Mortality readings were taken at 24 and 48 h. Mean percentage mortalities were used to plot regression lines. The LC_{50} values were noted.

Bioassay procedure on snails

Tests were carried out in five glass jars containing 500 ml of various dilutions of the final whole culture. Ten each of *Melanoides tuberculata, Physa waterlotti, Biomphalaria pfeifferi* and *Bulinus truncatus* were tested per dilution. These tests were replicated four times. The snails were fed with dried lettuce leaves. Mortality readings were taken at 24, 48, and 96 h.

Viable and spore counts

Viability and spore counts were determined before and after pasteurizing dilutions of the final whole culture (FWC). For these determinations, 10^{-5}, 10^{-6} and 10^{-7} dilutions of the FWC were plated in duplicate on nutrient agar and incubated for 48 h. Counts were taken from plates which gave 50-300 colonies. The mean for each dilution was taken.

Results and discussion

Preliminary bioassay of the *B. sphaericus* preparations conducted on four species of aquatic snails, including those from which the pathogens were isolated, yielded no mortality. These were *M. tuberculata* and *P. waterlotti*, from which the pathogens *B. truncutus* and *B. pfeifferi* were isolated. On the other hand, they all proved active against *Culex* and *Anopheles* larvae. Comparative larvicidal activity of the six strains on fourth instar *Cx. quinquefasciatus* larvae are shown in Table 1. These results indicated that all the strains possessed high larvicidal activity on *Cx. quinquefasciatus* larvae. The LC_{50} values (dilution of FWC) obtained ranged from 2.6×10^{-6} to 8.0×10^{-6} with IAB 482.2 proving the most active and IAB 481 showing the least activity. These values translate from 1.8×10^{3} to 8.0×10^{3} C.F.U. 1ml, and from 0.77×10^{3} to 2.0×10^{3} spores per ml. Thus all the strains seemed to possess similar

levels of activity. In all cases, the majority of the larvae succumbed within the first 24 h. When the exposure period was extended to 48 h the slopes of the regression lines increased only slightly (Table 1). This suggests that the maximum activity of the pathogens occurred within 24 h, and that after this period very little mortality occurred even when the exposure period was extended to 72 h.

The foregoing observation agrees with that made by Ramoska, Singer & Levy (1977). However, Mulla *et al.* (1984b), working with *B. sphaericus* strains on several species of mosquitoes, observed that *B. sphaericus* strains caused little or no mortality during the first 24 h period and that 48 h were needed for full expression of mortality. Since different strains were used in the above studies perhaps this feature varied with the strains.

Preliminary evaluation carried out on *An. gambiae* produced 100 per cent mortality with each pathogen on the larvae in the 10^{-5} dilution of the final whole culture within 48 h. *Aedes aegypti* on the other hand proved resistant to all the pathogens, no mortality was observed when they were exposed to 10^{-2} dilution of the various preparations. The low response generated by *Aedes* to *B. sphaericus* toxin has been observed by several investigators (Ramoska *et al.*, 1977; Mulligan, Shaefer & Miura, 1978; Lacey *et al.*, 1988). The resistance was originally thought to be due to inactivation of the toxin by midgut proteases of *Aedes* larvae (Mian & Mulla, 1983). Later studies by Davidson *et al.*, (1987), however, showed that gut extracts from *Ae. aegypti* were capable of activating the *B. sphaericus* toxin and that toxin treated with these extracts was not reduced in insecticidal activity as proposed by earlier investigators. This led them to hypothesise that the insensitivity of *Ae. aegypti* to *B. sphaericus* toxin occurred at the cellular level. In other studies, Davidson (1988, 1989) demonstrated that resistance of *Aedes* to *B. sphaericus* toxin was due to the failure of toxin to bind to midgut cells of *Aedes* larvae.

The fact that the pathogens were not active against the snails from which they were isolated but were highly active against mosquito larvae suggests that insecticidal *B. sphaericus* strains may not always be associated with organisms to which they are known to be pathogenic.

In two independent studies Weiser (1984) and Lysenko *et al.* (1985) similarly observed that insecticidal *B. sphaericus* strains isolated from a blackfly, grasshoppers and caterpillars, respectively, were not active against these insects but were highly active against mosquitoes. The latter authors suggested that such an association might merely be opportunistic or that the bacteria might act as secondary pathogens.

Colonies developed by the strains on nutrient agar showed typical colonial characteristics of *B. sphaericus,* being round, off-white with depression in the centre. Observation under phase contrast microscope revealed racket-shaped cells bearing terminal spores. Most of the spores were accompanied by double crystals. They all reacted positively to urea agar and Gram's stain. Based on these observations, the pathogens were provisionally identified as strains of *B. sphaericus.* Based on their flageller antigens, all of these strains were assigned to serotype H6 (de Barjac *et al.*, 1988). Also, all of them were placed in phage group 3 (Yousten, personal communication). According to him strains of this phage group are the most active.

All the known mosquito virulent bacteria have been isolated from insects. The above strains are the first reported insecticidal *B. sphaericus* strains isolated from organisms other than insects and they have been shown to be as active as the best studied strains, namely 1593, 2362 and 2297 (de Barjac *et al.*, 1988).

In the past, the irrational use of chemical pesticides for vector control and, sometimes, for purposes unrelated to vector control has resulted in the development of resistance in mosquitoes and other disease vectors. To counteract the effects of resistance, increasing doses of these chemicals have been used to a level where the cost of

vector control has risen beyond the means of developing countries. There is, thus, an obvious need to supplement or replace these chemicals. Results from these studies suggest that these bacterial strains have potential for the control of mosquitoes. This potential must be fully exploited.

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