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ABUNDANCE, DISTRIBUTION AND DIVERSITY OF *BACILLUS THURINGIENSIS* IN PAKISTANIAN ENVIRONMENT

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Four hundred and seventy six samples of soil, insect cadavers, wild animal dung, decaying leaves and grain dust collected from different ecological environments in Pakistan, were used as source material to separate 647 *Bacillus thuringiensis* isolates. The abundance and frequency of Bt in the various samples was found to be in the order of soil > grain dust > wild animal dung > bird droppings > others. Isolates were randomly selected to study pesticidal protein profile on SDS-PAGE and entomocidal properties in biotoxicity assays against *Heliothis armigera* (Lepidoptera) and *Anophelese stephensi* (Diptera). Several isolates were more efficacious against *Heliothis armigera* than the available reference strain.

Key words: Crystal protein, Lepidoptera, Diptera, *Bacillus thuringiensis*.

Introduction

Bacillus thuringiensis (Bt) is a gram-spore forming bacterium that produces a proteinaceous parasporal inclusion on sporulation [1]. *Bacillus thuringiensis* spores and/or inclusion bodies typically express entomocidal activity [2], and several strains are used on a global to combat larval forms of agriculturally important pests [3-5]. Although parasporal proteins constitute up to 30% of the sporal body-weight, the physiological functioning of such proteins is not precisely known. It has been proposed that the entomocidal activity of *B.thuringiensis* against target insect may be serendipitous [6].

Bacillus thuringiensis is widely distributed in the environmental and has been isolated from soil [7-11], dead insect, a sericulture environment (silk farm) [9,12], leaves of certain deciduous and coniferous trees [15] and stored products [12, 16-18]. *B.thuringiensis* harbors plasmid DNA, carrying several different insecticidal crystal protein (ICP) genes [19,20]. *B. thuringiensis* subsp. *Kurstaki* HD-1 is reported to harbour five ICP genes, *B. thuringiensis* subsp. *kurstaki* HD-73 a single ICP gene and *B. thuringiensis* subsp. *Israelensis* four ICP genes [21-23].

During the last two decades, over 42 crystal protein genes have been identified [6,23]. A comparison of their nucleotide sequences reveals that 13 genes specify a family of related insecticidal proteins. On the basis of host specificity, these 13 genes have been divided into four major classes and several sub-classes characterized by both the structural similarities and insecticidal spectra of the encoded protein [23]. The four major classes are lepidopteraspecific (1), lepidoptera and diptera-specific (II), coleoptera-specific (III) and diptera-specific (IV) genes. Recent discovery of Bt isolates larvicidal against sheep fleas and nematodes [6], suggest that other specificities exist in nature. It has been further observed that Bt isolates exhibiting the same serotype, but discovered from different parts of the world, show differing host range properties against insect pests. These observation, coupled with the need for environmentally safe pesticides, has encouraged a systematic search for novel host specificity and gene composition of newly isolated strains of *B. thuringiensis*. Discovery of new isolated will improve existing larvicidal potential of presently available *B. thuringiensis* isolates.

We have undertaken an elaborate programme of study to search systematically for Bt isolates with new host specificity and/or novel DNA sequences of the pesticidal genes. In this paper we report the collection of 476 samples which were used as source material to establish abundance, distribution and diversity of *B. thuringiensis* in Pakistan environment.

Materials and Methods

Chemicals and media. All the inorganic chemicals were purchaced from Merckshuchardt, Germay and all the organic chemicals were from Sigma Chemical Company, USA. All bacterial growth medium ingredients were from Difco. The protein assay kit was from Biord and Methyl-4-hydroxy-benzoate was from Fluka-Biochemica, Switzerland. Red bean powder and Baker's yeast for the *Heliothis* diet were purchased from the local market.

Bacterial strains. Standard *Bacillus thuringiensis* subsp. *Kurstaki*. type HD-1 was purchased from American Type Culture Collection. *B.thuringiensis* subsp.*israelensis* was a gift of the National Institute of Malarial Research and

TABLE 1. RELATIVE MORTALITY OF DIFFERENT INSECTS AFTER TREATMENT WITH *B. THURINGIENSIS* SPORE /CRYSTAL SUSPENSION OF KNOWN PROTEIN.

Isolates	Protein Profile Type	*H. Armigera* 200ng/mg	*A. Stephensi* 100µg/ml
C1.4	2	-	+++
D3.11	2	-	+++
D4.16	1	-	-
D4.10	2	++	++
D4.3	1	++	-
D1.1	2	-	++
F4.21	2	-	++
KC17.11	1	++	-
D4.4	1	-	-
RC1.5	1	++	-
KM3.1	2	-	+
KM3.3	3	-	-
KM7.3	3	-	+
KM7.5	3	-	-
KM7.6	3	-	-
KM9.5	3	-	++
BC3B6	3	-	+
BC14A8	3	-	++
SB2.3A	3	-	+
RC1.4	2	-	++
KC14.1	2	++	+++
JR6.3	1	+++	-
PR17.4	1	+++	-
PA1.2	2	-	+
3T	2	-	-
KC20.1B	2	-	++
RA2.12	1	-	-
kurstaki HD1	1	+++	-
israelensis	2	-	+++
israelensis	2	-	+++
PJ4.2	4	+++	Not determined
HW4.4	4	+++	Not determined
KM11.1	4	+++	Not determined
UC1.7	4	+++	Not determined
K2.1	5	+++	Not determined
BG2.6	5	++	+

No mortality + Mortality range 10-20% ++ Mortality range 40-60% +++ Mortality range 80-110%.

Training, Lahore, Pakistan. All *B.thuringiensis* cultures were grown on T3 medium (Tryptone 3g, Tryptose 2g, Yeast extract 1.5g, $MnCl_2$ 0.005g in 2.5mM Potassium Phosphate buffer, pH7.0) at 30°C unless stated otherwise.

Sample collection. Samples were collected from organically rich soil, animal dung, grain dust, bird droppings, dead insects and bat droppings from different ecological regions of Pakistan (Fig.1). Samples were taken from high altitude mountains, caves, beaches, forests, gardens, agricultural fields, grasslands, ruined soils, urban locations and archaeological excavations while grain dust samples were collected from provincial food storage godowns. The dead insects were collected from infested fields and godowns. In the case of soil samples the material was scraped off the surface with a sterile spatula and a 10-20g sample, was obtained from 2-5cm below the surface. These samples were stored in sterile plastic bags at 4°C before further processing.

Sample processing. Bt isolate were separated by the method of Martin and travers [8]. Bacterials colonies, appearing on T3-plates, were separated on the basis of morphological characteristics. Further identification of spore/crystal producing bacilli was done by staining with malachite green and basic fuchsin [24]. When viewed under the microscope, the spores appeared green and the vegetative cells and crystals turned deep pink. *Bacillus thuringiensis* isolates were stored as sporulated cultures on T3 medium slants and glycerol stocks.

Biotoxicity assays. For qualitative biotoxicity assays, crude spore/crystal preparations were grown on T3 plates. The spores/crystals were scraped of the plates and washed with sterile water [25]. Protein content of the suspension was estimated colorimetrically by using a Bio-rad Reagent dye [26].

The entomocidal activity of different Bt isolates was examined against the larvae of *Heliothis armigera* (Lepidopteran) and *Anopheles stephensi* (Dipteran). *H. armigera* was reared on an artificial diet developed in our laboratory (22.5% Red bean powder, 2.0% baker's yeast, 0.3% vitamin C, 0.1% sorbic acid, 0.15% formaldehyde, 1.28% agar and 0.2% methyl-4-hydroxy-benzoate). Spore/crystal suspensions were mixed with the artificial diet at approximately 200ng/mg solubilized protein concentration. Ten second instar larvae were fed the treated diet and mortality was monitored

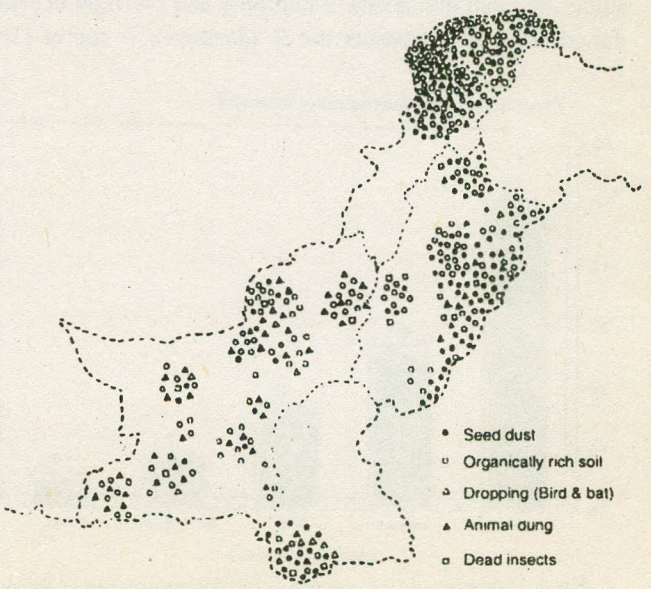

Fig. 1. Nature of samples and sampling sites in Pakistan.

after 5 days. All bioassays were done in triplicates. For biotoxicity assays against *Anopheles stephensi*, the spore/crystal concentration was adjusted at 100ng/ml with water. Ten fourth instar larvae were transferred to 100 ml protein suspension and mortality was monitored after 24 hr. Bioassays were done in triplicates.

Resolution of pesticide proteins. Parasporal crystal proteins were resolved on 10% SDS-PAGE [27]. Gels were stained with Coomassie stain (0.25% Coomassie brilliant blue R250, 45.5% methanol, 9% glacial acetic acid) and destained with several changes of destaining solution (25% ethanol, 7% galcial acetic acid).

Results and Discussion

476 Samples of soil, grain dust, animal dung, bird droppings, insect cadavers and decaying leaves were used as source material to separate *Bacillus thuringiensis* spore formers. Of these 49% were positive for *B. thuringiensis* and yielded 647 isolates. Among the other microorganims, *B. cereus* was the commonest contaminating species indicating its close taxonomic and ecological relationship with Bt [8,28,29]. The results in Fig. 2 show variation in Bt abundance in different samples from various locations. The soil samples were the richest source of crystal producers, showing an abundance of *B.thuringiensis* at 53% in comparison to only 17% in the US soils examined by Delucca *et al.*, [7] and 14% in the Philipine soils studied by Theunis *et al.*[8]. However, our results are in agreement with the findings of Martin and travers, [8] that Asain soils are extraordinarily rich in *B. thuringiensis*. Theunis *et al* [18] have reported that grain dust is the richest soure of Bt with 63% samples containing *B.thuringiensis* suggesting that low levels of humidity and UV light in abandoned grain mills favours the *B. thuringiensis* spores [18].

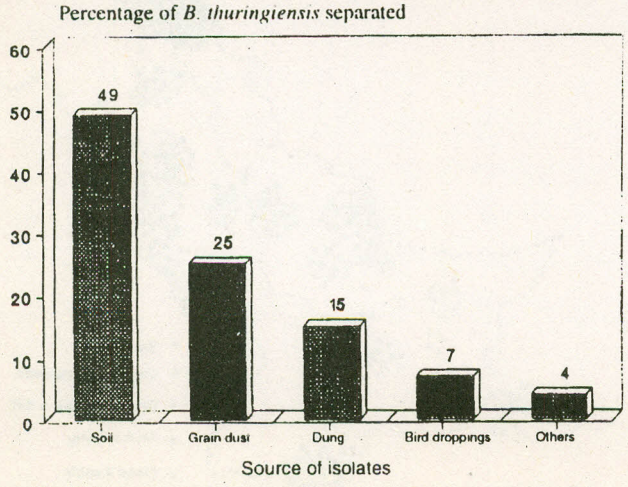

Fig. 2. Proportion of *B. thuringiensis* isolates separated from different samples.

However, we observed that only 25% of grain dust samples contained *B.thuringiensis*. Wild animal dung samples yielded 15% *B. thuringiensis*. Dead insects and sandy soils yielded only 10% *B. thuringiensis* and were the poorest sources of Bt (Fig. 2).

One fourth of the samples were analyzed by SDS-PAGE to obtain gel patterns for the pesticidal proteins in different Bt isolates separated from various source materials. It was observed that protein profiles of isolates from the same source were very similar, indicating a common origin of the various isolates. Interestingly, nine isolates (KM3.1, KM3.3, KM7.3, KM7.5, KM7.6, KM9.5, BC3b6, BC14A.8 and SB2.3a), separated from samples collected from the same region (northern areas of Pakistan), exhibited a protein profile distinctly different from the isolates from other regions of the country. According to Gonzalez *et al.*, [33] the observed diversity may be attributed to the fact that a vast majority of pesticidal genes are present on transmissible plasmides that allow their transfer among compatible cells to create novel combinations of toxin. Recently, however, Feitelson *et al.* [6] have proposed that bacterial gense have evolved in response to host target genes. Thus if an insect becomes resistant to one strain of *B. thuringiensis*, a selection pressure for a different strain may be created. That in turn may create pressure for a new insect variant.

The protein profiles of the various Bt isolates (Fig. 3) may be boardly classified five categories. The firs resembles Bt var. *kurstaki* HD-1, shown in Fig. 3A, lanes 3 and 4; Fig. 3B, lanes 4,5,7 and 8. The second resembles Bt var. israelensis (see Fig. 3A, lane 6; Fig. 3B, lane 6). The third category of protein profile consists of two prominent protein bands of approximately 70-72kd and 35-40kd (Fig. 3A, lanes 2 and 5, Fig. 3B, lane 3). The fourth category consists of several proteins in the range of 65-70kd (Fig. 3A, lanes 8 and 9; Fig. 3B, lane 10). The fifth and final category of protein profile was distincy different from all published patterns and showed a prominent band at approximately 50-55kd (Fig. 3, lane 8).

Thirty five Bt isolates, representing each category, were examined for insecticidal activity *H. armigera* (lepidoptera) and *A. stephensi* (diptera) as described in Materials and Methods. The results show that isolates designated as D4.10 and KC14.1, which exhibited a protein profile similar to that of Bt variety *israelensis*, were also active against *H. armigera* (Table 1). Since Bt *israelensis* is reported to be active only against diptera, it appears that isolates D4.10 and KC14.1 may contain a combination of CryI and CryIV genes. Isolates in category 4, for example PJ4.2 and UC1.7, were highly toxic against *H. armigera* showing 100% mortality at 200ng/mg of diet within 48 hr. The same level of activity was observed in 4-5 days with the reference standard HD-1. These

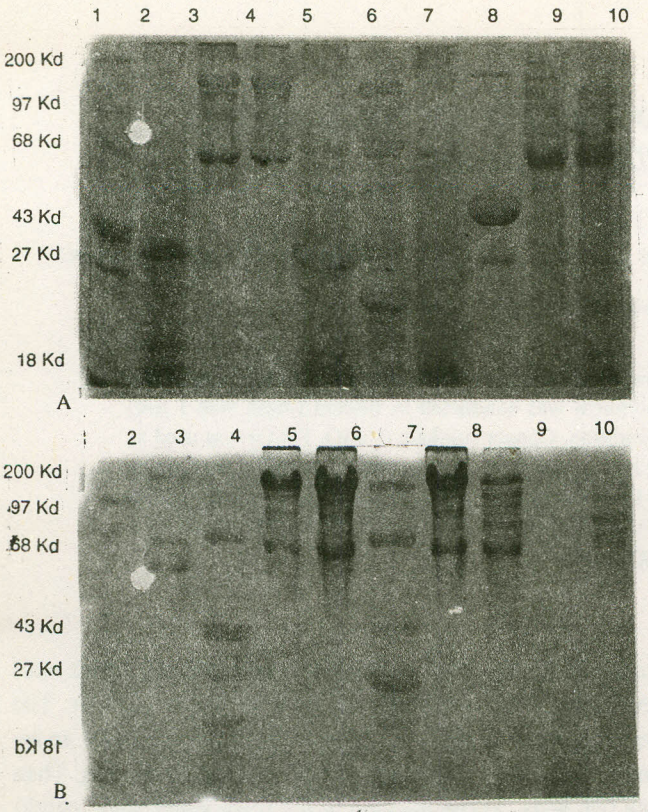

Fig. 3A & B. SDS-PAGE of crystal protein extracted from Bt strains isolated from Pakistan. (A) Lane 1: standard molecular marker (kd), lanes 2-10: isolates D4. 11, JR6.3, PR17.4, BC14A8, C1.4, KC14.1, BG2.6, KM11.1 and PJ4.2 respectively. (B) Lane 1: standard molecular marker (kd), Lanes 2-10: *kutdyski* HD1, KM9.5, RC1.5, INS2.13, D4.10, UC1.7, K2.1, Bt var. *israelensis* and JL1.1 respectively.

results suggest that the toxin combinations present in the local isolates are more potent against the target insects than the commercial strain HD-1 which contains five genes. Isolates BC14A8 and KM9.5, in category 3, exhibited moderate entomocidal activity against the dipteran *A. stephensi* and slight activity at high protein concentrations against the lepidopteran *H. armigera* (data not included). These results suggested the presence of cryll proteinsThis notion is further supported by the presence of a 71kd band (size of the CryllA protein) in the SDS polyacrylamide gel (Fig.3). Further characterization of the isolates to the gene level (work in progress) and future studies with solubilized crystal proteins and purified toxins will better define the potency of each isolate against the target insects and the toxin type.

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