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An improved enzyme-linked immunoassay for the detection and quantification of the entomocidal parasporal crystal proteins of *Bacillus thuringiensis* subspp. *kurstaki* and *israelensis*

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An improved and simplified enzyme-linked immunosorbent assay (ELISA) was developed for the detection and quantification of parasporal crystalline toxins from *Bacillus thuringiensis* subsp. *kurstaki*. The improved procedure involved pretreatment of the polystyrene cuvettes with glutaraldehyde before antibody coating. A direct comparison of treated and untreated cuvettes is provided. ELISAs were then used for the analysis of the entomocidal crystalline proteins in commercial and experimental formulations of *B. thuringiensis* subspp. *kurstaki* and *israelensis*.

Although immunoassay has been widely used in numerous fields, its application to the analysis of pesticides has been very limited. Interest in the immunochemical analysis of synthetic pesticides is rapidly escalating (Hammock & Mumma 1980; Wie & Hammock 1982), and an obvious and immediate application of immunoassay is the analysis of the protein toxins from *Bacillus thuringiensis*. In a classic study Winkler *et al.* (1971) used immunoelectrophoresis to optimize fermentation conditions while Andrews *et al.* (1980) significantly improved the speed of this assay. Although faster and more precise than bioassay, these methods are still somewhat slow and labour intensive.

Wie *et al.* (1982) first reported enzyme-linked immunosorbent assays (ELISAs) for the detection and quantification of the entomodical parasporal crystalline proteins of *B. thuringiensis* subsp. *kurstaki* and *israelensis* while Smith & Ulrich (1983) demonstrated the reliability of an ELISA for analysis of fermentation materials from *B. thuringiensis* subsp. *kurstaki*. It is important to analyze these protein toxins because the toxin of subsp. *kurstaki* is active on many lepidopteran insects (moths) whereas that of subsp. *israelensis* is more lethal to certain dipteran insects such as mosquitoes and blackflies. Moreover, the toxic proteins of both subspecies are biochemically and immunologically distinct (Krywienczyk & Fast 1980; Tyrell *et al.* 1981; Wie *et al.* 1982). Work of Thomas & Ellar (1983) demonstrates that the toxin of subsp. *israelensis* is toxic to several insect and vertebrate cell lines and to Balb. c mice as well. Recent work from this laboratory confirms the vertebrate toxicity of the solubilized toxin with an intraperitoneal lethal dose of $<0{\cdot}2$ mg/kg in

Swiss Webster mice. Moreover, the solubilized crystal proteins are toxic when injected into a wide variety of insect species (P.Y.K. Cheung, unpublished observations).

The potential of these *B. thuringiensis* toxins for controlling serious agricultural and medical pests with a minimum of environmental effects means that there is a great deal of interest in their direct commercialization and as targets for genetic engineering. Thus, for quality control, production optimization, efficacy evaluation, residue determination and genetic engineering as well as basic research, reliable, rapid analytical methods are needed for the toxic principles.

In this paper, significantly improved ELISAs for detecting and measuring insecticidal proteins of *B. thuringiensis* are described. The utility of these ELISAs is demonstrated by measuring insecticidal proteins in different commercial and experimental formulations and tank mixes of *B. thuringiensis.*

Materials and Methods

CULTURE CONDITIONS AND CRYSTAL PREPARATION

Bacillus thuringiensis subsp. *kurstaki, israelensis, alesti, tolworthi* and *berliner* were grown in a modified GYS medium containing (g/l): sodium citrate, 1·0; glucose, 1·0; $MgSO_4$, 0·2; $MnSO_4.H_2O$, 0·05; $(NH_4)_2SO_4$, 2 g; $K_2HPO_4.3H_2O$, 3·93; yeast extract, 2; final pH adjusted to 7·5 with conc. H_2SO_4. Following autoclaving a sterile solution of 0·08 g $CaCl_2$ in 10 ml of water is added to bring the total volume to 1 litre. Parasporal crystals were then separated from spores and cellular debris on Renografin gradients as described elsewhere (Tyrell *et al.* 1981). The crystals were dissolved in alkali as specified by Bulla *et al.* (1979, 1981) and dialysed overnight against 20 mmol/l NaH_2PO_4 at pH 7·5.

PREPARATION OF ANTISERA

Antisera were obtained by inoculating New Zealand white albino rabbits with a solution of purified crystal toxin from *B. thuringiensis* subsp. *kurstaki* and *israelensis* as described earlier (Andrews *et al.* 1980). Crude immunoglobulin fractions were obtained by precipitating 2 ml of antiserum with 40% saturated $(NH_4)_2SO_4$ at room temperature for 30 min and dialysing against 7 mol/l phosphate buffer containing 0·15 mol/l NaCl and 0·05% NaN_3 (pH 7·7).

PREPARATION OF ALKALINE-PHOSPHATASE-*B. thuringiensis* SUBSP. *berliner* CONJUGATES

Lyophilized protein toxin from *B. thuringiensis* subsp. *berliner* was stirred overnight with 2 ml of 0·2% Na_2CO_3 containing 0·02% NaN_3 at pH 12·0. It was then dialysed for 2 days against 0·1 mol/l phosphate buffer containing 0·15 mol/l NaCl, pH 7·4 with 2 changes per day. The toxic protein, 4·57 mg, was then coupled to 5 mg alkaline phosphatase in 2 ml of buffer by adding 15 μl of 25% glutaraldehyde. The mixture was stirred for 70 min at room temperature and then dialysed at pH 7·7. Following dialysis 0·4 ml of BSA solution (50 $\mu g/ml$) was added to stabilize the conjugate (Voller *et al.* 1978).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Indirect ELISA was carried out according to the method of Voller *et al.* (1980) with slight modifications and was used in this study for the detection of *B. thuringiensis* subsp. *israelensis* toxin. The toxin was diluted in 0·1 mol/l sodium carbonate buffer (pH 9·6) containing 0·02% NaN_3, and 250 μl was then added to each cuvette. The cuvettes were kept overnight at 4°C and washed three times with phosphate-buffered saline containing 0·05% Tween 20 and 0·02% NaN_3 (PBS-Tween). The assay procedure entailed two parts. First, 1 ml of the appropriately diluted rabbit antiserum was transferred to 13 × 100 mm culture tubes. To these antibody solutions were added inhibitor solutions of increasing concentration and they were incubated at 28°C overnight. The second step in the assay involved adding 250 μl of the above solutions to the *B. thuringiensis* subsp. *israelensis* toxin coated cuvettes which were incubated for 2 h at room temperature. After three washes with PBS-Tween, goat anti-rabbit Ig-alkaline phosphatase conjugate diluted in PBS-Tween (1/2500) was added and incubated at room temperature for 2 h. After another three washes, phosphatase activity was measured by adding 250 μl of *p*-nitrophenyl phosphate (1 mg/ml) in 10% die-

thanolamine buffer, pH 9·8. The reaction was stopped 30–40 min later by adding 3 mol/l NaOH and the colour intensity was measured at 405 nm in a Gilford EIA reader. Maximum binding as well as background binding were assessed by adding solvent only or no antibody, respectively, to the system.

For the direct ELISA, Gilford cuvettes were first treated with 0·2% glutaraldehyde in H_2O and incubated at 28°C. After 30 min the cuvettes were washed with distilled water and 150 μl of rabbit anti-*B. thuringiensis* subsp. *kurstaki* serum of different dilutions were added to the cuvettes, and they were then kept in a moistened chamber at 28°C overnight. The coated cuvettes then were washed three times with PBS-Tween. The *B. thuringiensis* subsp. *berliner*-alkaline phosphatase in PBS-Tween was then added to the cuvettes which were incubated for an additional 2 h at 28°C. For the preparation of standard inhibition curves, the inhibitors were added before the addition of toxin-enzyme conjugate. After three more washes, 250 μl of 1 mg/ml solution of *p*-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9·8, were then added to the cuvettes. The reactions were stopped 50–60 min later by addition of 50 μl of mol/l NaOH and the absorbance at 405 nm was monitored on a Gilford EIA reader.

SAMPLE PREPARATION

Because the crystal toxins were soluble in basic buffer at pH 12·0 (Bulla *et al.* 1981), a 0·2% Na_2CO_3 solution containing 0·02% NaN_3 was used to dissolve them. For the commercial formulations a total weight of 100 mg was suspended in 10 ml of Na_2CO_3 buffer and incubated at 28°C with vigorous shaking. Sonication to lyse cells was not required for optimal detection of toxin in either freshly harvested organisms or formulated materials. Very little difference in solubility was noted when the samples were incubated from 4 to 16 h. Following incubation the samples were centrifuged at 13 000g for 15 min, and the supernatant fractions were used to prepare serial dilutions for the ELISA.

Commercial formulations were generously provided by Sandoz Inc. (Homestead, Florida) and Abbott Laboratories (North Chicago, Illinois). Levels of toxins were also determined in standard unknown samples provided by the World Health Organization (WHO unknowns) of *B. thuringiensis* subsp. *israelensis*. These unknowns were sent to numerous laboratories throughout the world in an attempt to standardize bioassays. Antigenicity of pure crystal preparations and toxin levels in tank mixes were determined by dissolving a known weight of volume, respectively, in carbonate buffer. Tank mixes were prepared in the field by the US Forest Service for actual spray operations in the Lake States. Small aliquots of these tank mixes and the formulated materials used to prepare the tank mixes were numbered, frozen in dry ice and shipped to U.C. Davis for blind analysis.

REAGENTS

Alkaline phosphatase conjugated goat anti-rabbit IgG was obtained from Miles Laboratory (Elkhart, IN). Normal rabbit immunoglobulin was purchased from Antibodies Incorporated (Davis, CA). Alkaline phosphatase (1100 units/mg) type VII from bovine intestine and glutaraldehyde were purchased from Sigma Chemical Company (St. Louis, MO).

INSECT BIOASSAYS

The concentration needed to kill 50% (LC_{50}) of the early fourth instar mosquito larvae of *Aedes aegypti* (strain Bora-Bora from Polynesia) was determined by World Health Organization approved methods. The bioassays were standardized using the Institut Pasteur Standard 1978 (IPS 78) which has an arbitrarily defined potency of 1000 ITU/mg (International Units; de Barjac & Larget 1979).

Results and Discussion

Development of a practical direct ELISA for toxins from *Bacillus thuringiensis* requires the optimization of conditions for coating the anti-toxin antibody to the solid phase (cuvette). Initial studies using untreated cuvettes coated with a fixed concentration (1/100) of anti-*B. thuringiensis* subsp. *kurstaki* serum showed that antibody-coated cuvettes can be used to develop an ELISA for the toxins (Table 1). The absorbance at 405 nm was directly proportional to the concentration of *B. thuringiensis* subsp. *berliner*-alkaline phosphatase used in the assay. Analogous experiments using nonimmune (normal)

Table 1. Direct enzyme-linked immunosorbent assays using rabbit anti-*B. thuringiensis* subsp. *kurstaki* serum and subspecies *berliner*-alkaline phosphatase conjugate*

Dilution of toxin coupled alkaline phosphatase	Absorbance at 405 nm		
	Normal plates	BSA and Glutaraldehyde† pretreated	Glutaraldehyde‡ pretreated
1/100	1·53	1·69	>3
1/200	1·25	1·04	>3
1/400	0·97	0·90	2·8
1/800	0·92	0·60	1·73
1/1600	0·79	0·50	1·34

* The Gilford cuvettes were coated with 150 μl of a 1/100 dilution of rabbit anti-*B. thuringiensis* subsp. *kurstaki* serum for 2·5 h at 28°C (see Materials and Methods). After washing with PBS-Tween, serial dilutions of *B. thuringiensis* subsp. *berliner*-alkaline phosphatase were added and incubated for 2 h. After subsequent washes, 1 mg/ml of *p*-nitrophenyl phosphate was added and the reading at 405 nm was taken 60 min later.

† Cuvettes were first treated with 150 μl of 0·2 mg/ml BSA and subsequently fixed with 0·2% of glutaraldehyde.

‡ Cuvettes treated with 150 μl of 0·2% glutaraldehyde only.

rabbit serum and either treated or untreated cuvettes showed that little if any of the enzyme-toxin conjugates adhered to the cuvettes. Since it was reported that polystyrene plates that were treated with BSA and subsequently fixed with glutaraldehyde facilitated the binding of antibody to the solid phase (Pestka *et al.* 1981), such experiments were then carried out as described and some of the results using anti-*B. thuringiensis* subsp. *kurstaki* antiserum are shown in Table 1. Although comparable absorbance was obtained for normal and BSA- and glutaraldehyde-treated cuvettes at the higher antigen-enzyme concentrations, a significantly lower absorbance was found for BSA-glutaraldehyde-treated cuvettes at lower antigen-enzyme concentrations. It appears that BSA treatment in our system did not offer an advantage in facilitating the antibody-antigen interaction. On the other hand, cuvettes treated with glutaraldehyde alone gave a higher absorbance in all the antigen-enzyme concentrations used in the direct ELISA which in turn increased the sensitivity of the assay and conserved toxin-coupled alkaline phosphatase. When different dilutions of anti-*B. thuringiensis* subsp. *kurstaki* serum and a fixed concentration of *B. thuringiensis* subsp. *berliner*-alkaline phosphatase conjugate were used in the ELISA, glutaraldehyde-treated cuvettes gave a > two-fold increase in the absorbance at serum dilutions of 1/1600–1/3200 as compared with the untreated cuvettes (Fig. 1). When cuvettes were coated with the antibody first and then treated with 0·2 mg/ml solution of BSA, slightly lower absorbances were observed with all antibody concentrations used. Thus, in this study subsequent blockage of active sites on the plate only slightly decreased assay background while causing a substantial decrease in assay sensitivity. As mentioned earlier, non-specific binding of the enzyme-toxin to the cuvette is unlikely, and this notion is reinforced by the observation that the absorbance decreased to the background level with the increasing dilution of the antiserum. The specificity of the assay was also shown by the fact that there was no detectable enzymatic activity in glutaraldehyde-treated cuvettes when rabbit anti-*B. thuringiensis* subsp. *israelensis* toxin serum was used in place of the anti-subspecies *kurstaki* antibodies. This experiment also confirms our previous observation that no antigenic cross-reaction in ELISA assays was found between the *B. thuringiensis* subsp. *israelensis* and the other *B. thuringiensis* toxins including those from subsp. *kurstaki, tolworthi, alesti* and *berliner*.

In a separate experiment, cuvettes were treated with 0·02% glutaraldehyde and incubated at room temperature for various time intervals (5 min to 2 h) before washing. The antiserum used was a 1/2500 dilution of rabbit anti-*B. thuringiensis* subsp. *kurstaki* and the enzyme was a 1/2000 dilution of *B. thuringiensis* subsp. *berliner*-alkaline phosphatase conjugate. The results indicate that with this system the length of incubation with glutaraldehyde is not critical since no significant difference in absorb-

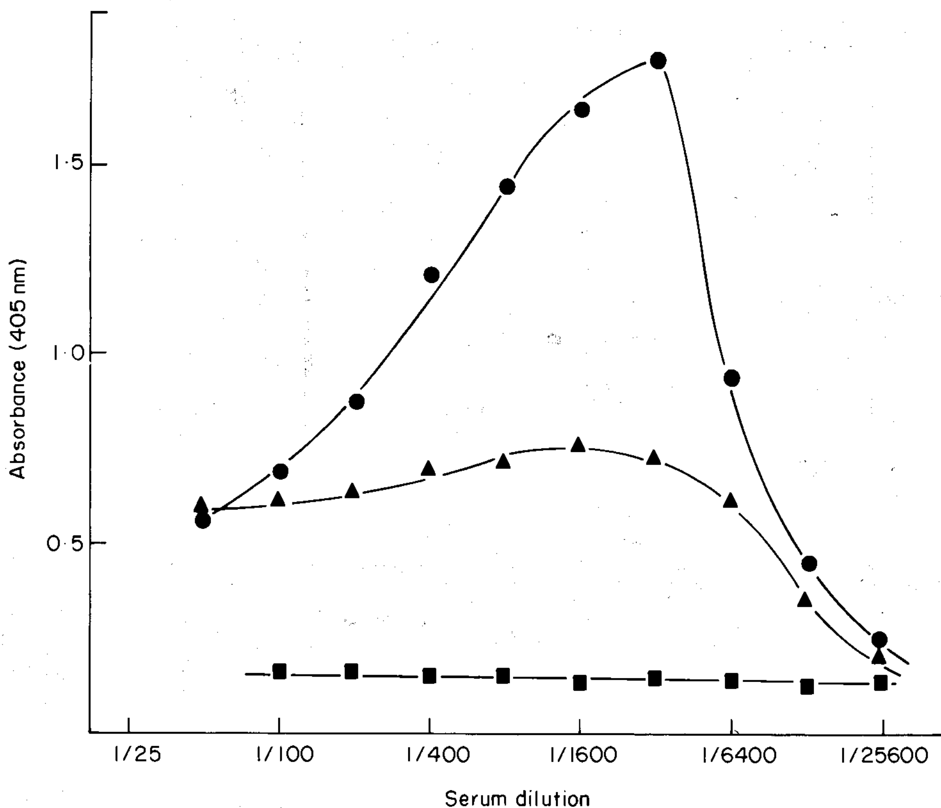

Fig. 1. The effect of glutaraldehyde pre-treatment of polystyrene cuvettes in direct ELISAs. The extend of binding of the alkaline phosphatase-coupled *B. thuringiensis* subsp. *berliner* toxin to the ELISA cuvettes is indicated by the absorbance at 405 nm assayed at multiple serum dilutions. ●, Cuvettes treated with 0·2% glutaraldehyde and coated with the anti-subspecies *kurstaki* antibody; ▲, nontreated cuvettes coated with anti-subspecies *kurstaki* antibody; ■, 0·2% glutaraldehyde treated cuvettes coated with anti-subspecies *israelensis* antibodies.

ance was observed. Overnight incubation with glutaraldehyde yielded a slight increase in absorbance. This result further simplifies the use of the glutaraldehyde method since the timing of glutaraldehyde pre-treatment of the plates is not especially critical. In other studies (results not shown) antibody binding to the glutaraldehyde-treated plates was shown to occur rapidly and then very slowly increase when the coating antigen was held for several days in the plates at 5°C. The glutaraldehyde treatment appears useful with a variety of solid phases including Falcon plates and polystyrene balls. In all cases tested, glutaraldehyde pretreatment increased the effective titre of the antibody used for coating the plates as well as the sensitivity of the resulting ELISA. This effect may be due, in part, to the glutaraldehyde treated plates favouring an effective orientation of the coating antibody on the plate surface. It is not universally applicable however to proteins other than antibodies. In some systems it conserved coating antigen while in others glutaraldehyde pretreatment had little if any effect on indirect ELISAs.

The bell-shaped curve shown in Fig. 1 may explain why some workers failed to see a significant improvement in their immunoassay when using glutaraldehyde. If only high serum concentrations had been evaluated in this study, glutaraldehyde treatment would not have demonstrated an advantage. The reasons for the shape of the curve in Fig. 1 are not clear. Possibly at high antibody concentrations the glutaraldehyde is able to introduce cross links between individual antibody molecules and thereby prevent them from subsequent effective

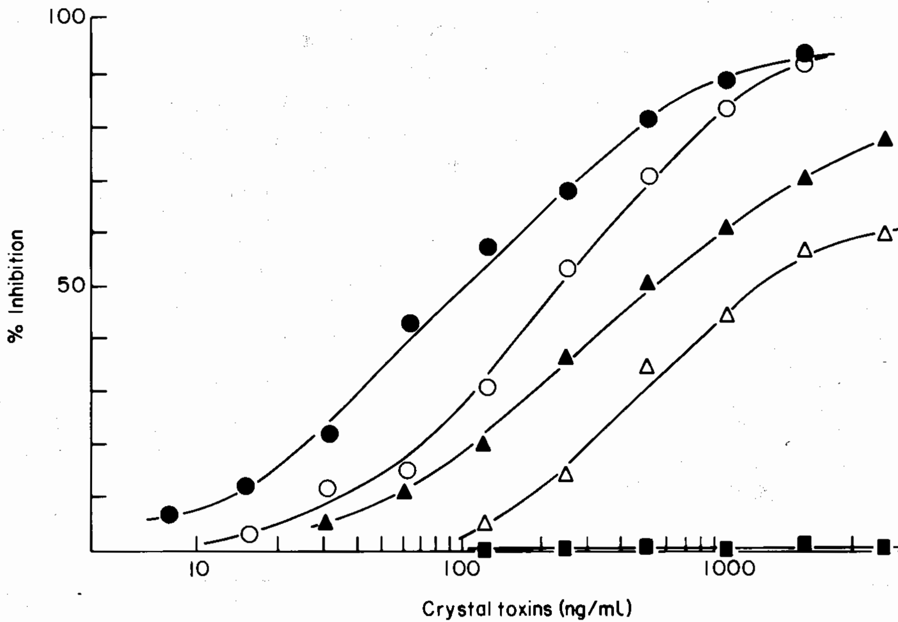

Fig. 2. Inhibition of binding of *B. thuringiensis* subsp. *berliner*-alkaline phosphatase by crystal toxins from several subspecies of *B. thuringiensis* (ng/ml). The direct ELISA employed glutaraldehyde pretreated rabbit anti-*B. thuringiensis* subsp. *kurstaki* cuvettes. ●, *B. thuringiensis* subsp. *kurstaki*; ○, *B. thuringiensis* subsp. *berliner*; ▲, *B. thuringiensis* subsp. *tolworthi*; △, *B. thuringiensis* subsp. *alesti*; ■, *B. thuringiensis* subsp. *israelensis*.

binding with the antigen. It has been speculated that the effectiveness of the glutaraldehyde treatment stems from the stabilization of the antibody in spite of strong interactions with the solid phase (Parsons 1981).

Inhibition studies were carried out to determine the capacity of different *B. thuringiensis* toxins to prevent the binding of *B. thuringiensis* subsp. *berliner*-alkaline phosphatase to the anti-*B. thuringiensis* subsp. *kurstaki* antibody coated cuvettes. The results shown in Fig. 2 are expressed as the percent inhibition of the absorbance observed using a standard amount of toxin-enzyme conjugate plotted against a variable concentration of toxins. Two criteria were used to compare the cross reactivity of various pure crystals. First, the concentration of solubilized crystal which reduced absorbance by 50% indicated the concentration of added toxin which caused a 50% displacement of the alkaline phosphatase coupled toxin from the antibody coated cuvettes (I_{50}). This I_{50} is related to the affinity of the toxin tested for the antibody. Second, the maximum displacement which could be obtained indicates the antigenic determinants which the various toxins have in common.

As expected, the *B. thuringiensis* subsp. *kurstaki* toxin gave the strongest displacement with an I_{50} at 105 ng/ml and a maximum displacement of 94%. The toxin from *B. thuringiensis* subsp. *berliner* also gave significant displacement with an I_{50} of 220 ng/ml and a 94% maximum displacement. Toxins from *B. thuringiensis* subspp. *tolworthi* and *alesti* were found to contain fewer antigenic determinants than the toxin from subsp. *kurstaki* and therefore could not completely displace the toxin-enzyme conjugate. On the other hand, the toxin from *B. thuringiensis* subsp. *israelensis* appears to be unique since it did not appear to cross-react with the other toxins (Fig. 2). Similar results were also obtained in the indirect ELISA as described in the previous study (Wie *et al.* 1982).

It should be recalled, however, that ELISAs are competitive assays. Thus, the *B. thuringiensis* subsp. *israelensis* toxin could have some antigenic determinants in common with the *B. thuringiensis* subsp. *kurstaki* toxin, but if the average K_d with the antibody population was signifi-

cantly higher, inhibition would not be observed in a direct or indirect ELISA as performed here.

The direct ELISA was then used to determine the possible contamination of *B. thuringiensis* subsp. *kurstaki* in several *B. thuringiensis* subsp. *israelensis* experimental and commercial formulations. Toxins from *B. thuringiensis* subsp. *israelensis* were determined by an indirect ELISA developed in this laboratory using specific rabbit anti-*B. thuringiensis* subsp. *israelensis* antisera which do not recognize antigenic determinants of toxins derived from subspecies *kurstaki* and its related strains (Wie *et al.* 1982). As shown in Table 2, WHO IPS 78, Sandoz T15-2A and Abbott ABG 6108D have similar amounts of *B. thuringiensis* subsp. *israelensis* toxin, whereas only negligible amounts of *B. thuringiensis* subsp. *kurstaki* determinants were found in both WHO IPS 78 and Sandoz T15-2A. The formulation from Abbot, however, showed a significant level of this toxin. It is possible that this contamination resulted from sequential use of a fermenter to produce *B. thuringiensis* subsp. *kurstaki* and *israelensis* since it is difficult and possibly not necessary to sterilize equipment used to produce spore-forming bacteria. However, researchers should be very cautious when using commercial formulations to study species specificity. Several WHO unknown samples which were reported to contain *B. thuringiensis* subsp. *israelensis* toxin were also included in Table 2 and they were found to contain high levels of the larvacidal *B. thuringiensis israelensis* except unknown D which had mainly the subspecies *kurstaki* determinants rather than subspecies *israelensis* determinants. Possibly *B. thuringiensis* subsp. *kurstaki* was used deliberately to dilute the *B. thuringiensis* subsp. *israelensis* when the standard unknowns were prepared.

LC_{50} values obtained on *Aedes aegypti* larvae according to WHO methods were compared with the toxin levels detected by direct ELISA. For instance, the WHO IPS-78 standard with an LC_{50} value of 0·24 mg/l had 115 μg/l of subspecies *israelensis* toxin and undetectable levels of subspecies *kurstaki* toxin. The unknowns were all derived from diluted sample A and the results indicate that LC_{50} values correlated with the experimentally determined *B. thuringiensis israelensis* toxin levels. Thus, WHO unknown D which had an LC_{50} of > 100 mg/l had negligible subspecies *israelensis* toxin (0·1 μg/ml) and large amounts of subspecies *kurstaki* toxin (240 μg/ml). Samples B, D and E all had subspecies *israelensis* toxin in addition to *kurstaki* toxins (Table 2).

Similarly, using direct ELISA, the amount of subspecies *kurstaki* toxin in commercial tank mixes prepared from Dipel 14-706 CF was determined and found to be proportional to the amount of *B. thuringiensis* subsp. *kurstaki* formulation added to the sample in a blind experiment. When identical formulation ingredients without *B. thuringiensis* were assayed, false positives were not found and the ingredients did not interfere with the positive ELISAs.

The present study demonstrates that a simple

Table 2. Determination of toxin content (μg toxin/mg formulation) from different formulations using ELISA

Formulation*	*B. thuringiensis* subsp. *kurstaki*†	*B. thuringiensis* subsp. *israelensis*‡	Mosquito toxicity LC_{50} (ppm)§
IPS-78	UD	11·5	0·24
Sandoz T15-2A	UD	10·2	NE
Abbott ABG 6108D	3	9·4	NE
WHO unknown A	16·4	18·5	0·33
WHO unknown B	53	16·5	0·46
WHO unknown D	240	0·1	>100‖
WHO unknown E	80	9·3	0·73

* See text for a discussion of formulation.
† Detected by direct ELISA as described in Materials and Methods.
‡ Detected by indirect ELISA (Wie *et al.* 1982).
§ Methods followed were given in WHO correspondence T16/181/V2/01 and VBC/80.0 and used fourth instar larvae of the Bora Bora strain of *Aedes aegypti*.
‖ At 100 mg/l there was only 35% mortality.
UD, undetectable; NE, not established.

direct ELISA can be adapted for determining the level of insecticidal parasporal proteins of *B. thuringiensis* subsp. Such assays are more compatible with field use because of their enhanced speed and simplicity. The use of glutaraldehyde pre-treatment of the cuvette enhanced the binding of antibody molecules to the solid phase. Although glutaraldehyde has been previously proposed for covalent coupling of proteins to polymeric surfaces (Barrett 1977; Parsons 1981), this procedure has been sporadically used for solid phase immunoassays. Possibly the reluctance to use this procedure stemmed from the reported complexity of the coupling procedure and the lack of a clear demonstration that the technique offered advantages over more classical coating techniques (Parsons 1981). In this study the glutaraldehyde pre-treatment used was very simple and resulted in more sensitive assays while conserving the coating antibody. Thus this technology may prove useful in the further development of a variety of solid phase immunoassays.

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