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Biosafety of Recombinant and Wild Type Nucleopolyhedroviruses as Bioinsecticides

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Abstract: The entomopathogenic *Autographa californica* (Speyer) nucleopolyhedrovirus (AcMNPV) has been genetically modified to increase its speed of kill. The potential adverse effects of a recombinant AcMNPV (AcAaIT) as well as wild type AcMNPV and wild type *Spodoptera littoralis* NPV (SlNPV) were studied. Cotton plants were treated with these viruses at concentrations that were adjusted to resemble the recommended field application rate (4 x 10^{12} PIBs/feddan, feddan = 4,200 m^2) and 3rd instar larvae of *S. littoralis* were allowed to feed on the contaminated plants. SDS-PAGE, ELISA, and DNA analyses were used to confirm that larvae that fed on these plants were virus-infected. Polyhedra that were purified from the infected larvae were subjected to structural protein analysis. A 32 KDa protein was found in polyhedra that were isolated from all of the viruses. Subtle differences were found in the size and abundance of ODV proteins. Antisera against polyhedral proteins isolated from AcAaIT polyhedra were raised in rabbits. The terminal bleeds from rabbits were screened against four coating antigens (i.e., polyhedral proteins from AcAaIT, AcAaIT from field-infected larvae (AcAaIT-field), AcMNPV, and SlNPV) using a two-dimensional titration method with the coated antigen format. Competitive inhibition experiments were conducted in parallel to optimize antibody and coating antigen concentrations for ELISA. The IC_{50} values for each combination ranged from 1.42 to 163 µg/ml. AcAaIT-derived polyhedrin gave the lowest IC_{50} value, followed by those of SlNPV, AcAaIT-field, and AcMNPV. The optimized ELISA system showed low cross reactivity for AcMNPV (0.87%), AcAaIT-field (1.2%), and SlNPV (4.0%). Genomic DNAs isolated from AcAaIT that were passaged in larvae of *S. littoralis* that were reared in the laboratory or field did not show any detectable differences. Albino rats (male and female) that were treated with AcAaIT, AcMNPV or SlNPV (either orally or by intraperitoneal injection at doses of 1 x 10^8 or 1 x 10^7 PIBs/rat, respectively) appeared to be healthy and showed increased body weight at 21 days posttreatment. The effect of virus administration on hematological, serum biochemical, and histopathological parameters were determined. Slight to moderate differences were observed in most of the hematological parameters. Specifically, serum proteins were decreased markedly in female rats treated orally with SlNPV, and in male rats injected with AcAaIT. SDS-PAGE analysis also showed some changes in serum protein profiles. No marked changes in acetylcholine esterase (AChE) activity were found. Changes in serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, creatinin, and urea were also observed. Immunohistochemical observation of tissues from stomach, intestine, liver, kidney, brain, spleen, and lung also showed slight changes. Fish (*Tilapia nilotica*) were also exposed to AcAaIT, AcMNPV or SlNPV by incorporating each of the viruses into diet (1 x 10^9 PIBs/group). No mortality was found in treated or untreated fish during the experimental period (28 days). Macrophage phagocytic activity of fish head kidney cells increased with time, reaching maximum values at 180 min for both treated and control fish.

Keywords: Nucleopolyhedrovirus, recombinant baculovirus, bioinsecticide, biosaftey, *Spodoptera littoralis*, toxicity, histopathology, immunotoxicity, macrophage phagocytosis

Introduction

The effective control of pest insect populations is an essential prerequisite for producing food and commodities for man and domestic animals. Chemical insecticides are highly effective for maintaining pest insect populations below the economic threshold, however, problems associated with the inappropriate use of chemical pesticides such as environmental contamination, the development of insecticide resistance, and the elimination of beneficial or non-target insects must be considered. Thus, the search for safer agents and methods for plant protection has intensified in recent years. The use of biological insecticides, either as sole use biopesticides or in combination with other agents and methods in an integrated pest management system has gained momentum during the last 20 years.

Viruses comprise an important class of insect pathogen. The benefits of using insect baculoviruses for biological control have been studied for several years. The wild type baculovirus, however, has a long killing time in comparison to chemical pesticides. Molecular biological technology has been used to overcome this and other problems associated with the wild type baculovirus (reviewed in [1, 2]). The baculovirus type species, *Autographa californica* multiple nucleoplyhedrovirus (AcMNPV), has been commonly used as a model virus to test the efficacy of pesticidal gene cassette constructs under laboratory and field conditions for improvements in pesticidal properties. The initial strategies for improving the pesticidal activity of the baculovirus involved the insertion of genes that regulate the physiology of the target insect into the baculovirus genome [3, 4]. Insertion of an insect-selective toxin gene (e.g., *aait* from North African scorpion *Androctonus australis*) into the baculovirus genome has been one of the most effective strategies for improving insecticidal efficacy [5, 6].

The concept of biosafety is a concern associated with the use of genetically modified organisms (GMOs) and, more generally, with the introduction of nonindigenous species into natural or managed ecosystems. A relatively new concept in agricultural research, biosafety tempers the adoption of a new technology by carefully considering its potential effects on human health and the environment in general. In a series of regional policy seminars organized by ISNAR's Biotechnology Service (IBS), biosafety was designated a high priority for countries in Southeast Asia, Africa, and Latin America, regardless of their level of indigenous technical capacity. Building competence in biosafety is thus strategically important for successfully integrating biotechnology into agricultural research. Biosafety is achieved by assessing and managing environmental risks, evaluating potential ecological consequences, and weighing these against potential benefits. A step-by-step, case-by-case approach fosters a deliberate, informed, and environmentally responsible use of biotechnology. Policymakers begin by naming a lead agency under which biosafety policy is to be implemented. The next step is usually to draft biosafety guidelines that provide a framework for administrative procedures and decision-making regarding the appropriate use of GMOs [7].

The aim of the present study was to get results that would provide a database for establishment of future environmental risk assessment protocols and guidelines for baculovirus pesticides in the local environment. This will enable the competent authorities to make informed decisions regarding GMOs and achieve biosafety protocols to protect man and biological diversity from the potential risks of modern biotechnology-based pest controlling agents.

Materials and Methods

Test Insects

A laboratory colony of *Spodoptera littoralis* (Boisd.) was obtained from the Institute of Plant Protection, Agricultural Research Center, Ministry of Agriculture, Dokki, Giza, Egypt. A field colony of *S. littoralis* was collected as egg masses from cotton fields, Sharkia province, Egypt. The insect larvae were kept at 25°C, 60-70% relative humidity on a 14:10 h day:night photoperiod. Larvae were reared on modified semi-synthetic bean diet [8] consisting of 500 gm white beans, 150 gm brewer's yeast, 10 gm ascorbic acid, 5 gm methyl-p-hydroxy benzoate, 2 mg sorbic acid, 30 gm agar, 10 ml formalin, and 1,200 ml distilled water.

Test Animals

Rat: Male and female white rats, *Rattus norvegicus*, 100-120 days old with average weight of 100 ± 10 gm, were used for acute oral and injection toxicity studies.

Rabbits: New Zealand white rabbits, with average weight of 1,500 ± 100 gm, were used for raising antibody.

The rats and rabbits were obtained from the test animal farm, Faculty of Veterinary Medicine, Zagazig University, and were housed separately in metal cages with free access to food (pellet ration). The animals received a complete health ration during the experiment period.

Fish: *Tilapia nilotica* (ca. 10 gm/fish) were used in the macrophage phagocytosis studies. The fish were obtained from Abbassa fishponds (Abbassa Research Center, Sharkia Province, Egypt). Prior to use, the fish were acclimatized in bioassay tanks containing aerated chlorine-free tap water under laboratory conditions (i.e., natural photoperiod and temperature, 20ºC, and access to a commercial dry food) for two weeks.

Test Viruses

AcMNPV and the construction of AcAaIT (a recombinant AcMNPV expressing AaIT) are described in [5]. *S. littoralis* NPV (SlNPV) was obtained from the Entomovirology Laboratory, Cairo University.

Propagation and Purification of Polyhedral Inclusion Bodies (PIBs)

Third instar larvae of *S. littoralis* were inoculated with AcMNPV, AcAaIT or SlNPV by feeding them on modified semi-synthetic diet treated with virus at a concentration of 1 x 10^4 PIBs/mm^2. PIBs were isolated and purified from larval cadavers as described in [9]. In brief, larvae were homogenized in distilled water and the suspension was filtered through cheesecloth. The filtrate was then centrifuged at 1,000 rpm for 15 min, the pellet was resuspended in 0.5% sodium dodecyl sulfate (SDS) and 0.1% sodium deoxycholate, and incubated at 37°C for 2 h. The suspension was then filtered through two layers of cheesecloth and centrifuged at 1,000 rpm for 15 min. The pellet was then resuspended in 30 ml of distilled water and centrifuged at 1,000 rpm. This process was repeated three times. The virus preparations were mixed for 1 min in a sonicator to obtain a uniform suspension, and then layered onto a 40 to 66% (w/w) sucrose density gradient and centrifuged using a Beckman SW28 rotor at 15,000 rpm for 1 h. After centrifugation the virus band was removed from the gradient with a Pasteur pipette, washed in distilled water, and centrifuged at 8,000 rpm for 15 min. The pellet was resuspended in sterile distilled water to give a final concentration of approximately 8.5 x 10^8 PIBs/ml. PIB numbers were quantified using a counting chamber (Brightline Haemocytometer).

Propagation of AcAaIT in Larvae of *S. littoralis* Under Cotton Field Conditions

During the spring of 2004, cotton seedlings were planted (10 rows, with roughly 30 plants (10 groups of three plants) per row) in an experimental field of Zagazig University. In June of 2004, when the plants were 9 weeks old and approximately 30 cm in height, twelve groups of plants (3 plants per group) in the middle of the field were sprayed with an aqueous solution of AcAaIT polyhedra. The virus concentration was adjusted to resemble the recommended field rate of 4 x 10^{12} PIBs/feddan (feddan = 4,200 m^2). Subsequently, 15 healthy 3rd instar *S. littoralis* were released at various positions of each group of plants. The plants were then covered with a mesh cage that was buried 20 cm into the soil in order to prevent escape of the larvae and to prevent other insects from feeding upon the experimental plants. Untreated plants were used as a control. The larvae were observed daily. All of the larvae were collected at 7 days post release. The average temperature and relative humidity values were 30ºC and 57%, respectively, during this period.

Analysis of Viral Structural Proteins by SDS-PAGE

Polyhedra and occlusion derived virions (ODVs) were prepared according to [10]. The polyhedra were dissolved by incubation in DAS buffer (0.3 M Na_2CO_3, 0.5 M NaCl, and 0.03 M EDTA, pH 10.5) at 37°C for 30 min. Undissolved polyhedra and heavy debris were pelleted by centrifugation at 1,500g for 5 min. The supernatant was then centrifuged at 76,690g for 30 min. to pellet the ODVs, and the ODVs were resuspended in a small volume of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and layered onto a 30-60% (w/v) sucrose gradient in TE. After centrifugation at 50,000g for 2 h, the bands containing ODVs were collected and washed twice with TE by centrifugation (at 76,690g for 30 min). Polyhedron and ODV proteins were analyzed by SDS-PAGE according to the method of [11]. The ODV and polyhedron proteins were boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, and 10% glycerol, pH 6.8), and electrophoresed through 12.5% SDS-polyacrylamide gels. Proteins were visualized by overnight staining in a solution containing 25% (v/v) methanol, 10% (v/v) acetic acid, and 0.12% (w/v) Comassie Brilliant Blue R250, and destained in 7.5% (v/v) methanol and 5% (v/v) glacial acetic acid. Low range of SDS-PAGE molecular weight standards were used for size comparison.

Raising Polyclonal Antibodies against AcAaIT polyhedrin and ELISA

Antigen Preparation

AcAaIT polyhedra were purified from moribund larvae as described above. Polyhedrin protein was purified from AcAaIT polyhedra following dissociation in 0.1 M Na_2CO_3 at pH 11 for 30 min. Undissolved polyhedra and heavy debris were pelleted by centrifugation at 1,500g for 5 min. The supernatants were then centrifuged at 76,690g for 30 min to pellet the ODVs. The final supernatant was taken and adjusted to pH 7.3 with 2 N HCl. Protein concentration was determined by Bradford's reagent using a commercially available kit.

Production of Antisera

Polyclonal antibodies for the polyhedrin protein of AcAaIT were raised in albino rabbits according to [12]. The first subcutaneous injection was composed of 0.5 ml of antigen (containing ca. 1 mg of polyhedrin) and 0.5 ml of Found's Complete Adjuvant. The second and third injections were made by mixing the antigen (0.5 ml) with Found's Incomplete Adjuvant (0.5 ml). Serum samples were taken 1 week after the second and the third injections, and the titer of antibody was measured.

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked immunosorbent assay (ELISA) and competitive ELISA were performed according to [13]. AcAaIT polyhedrin was used as the coating antigen. The coating antigen was diluted in coating buffer (0.5 M sodium carbonate/bicarbonate, pH 9.6) at concentrations of 20, 10, 5, 2.5 and 1.25 µg/ml and added to the microtitre plates (100 µl/well). The plates were sealed with adhesive plate sealer and incubated at 4ºC overnight. The coated

plates were washed 3-5 times with phosphate buffered saline tween azide (PBSTA) to remove unbound coating antigen, then serial dilutions of antiserum in PBSTA were added at 100 µl/well, followed by incubation at room temperature for 2 h. The plates were again washed 3-5 times with PBSTA. Goat anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBSTA (1:2,500) was added at 100 µl/well and incubated at room temperature for 2 h. After another 3-5 times of washing, phosphatase activity was measured by adding 100 µl/well of *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine buffer, pH 9.8, and incubated for 30 min at room temperature. The absorbance readings were recorded by using a Microplate Autoreader (Bio-TEK Instruments EL311) at a single wavelength of 450 nm. The data were collected and analyzed by linear regression analysis using the Immunosoft Program (Dynatech). Maximum binding and background binding antibody concentrations were measured where neither analyte nor antibody were added to the system.

Assay Optimization

Optimal concentrations for the coating antigen and antisera were determined by screening in a two-dimensional titration as described above and used in inhibition studies. After comparison of the competitive inhibition curves for the antisera and four coating antigen combinations (i.e., polyhedrin proteins from AcAaIT, AcAaIT from field-infected larvae (AcAaIT-field), AcMNPV, and SlNPV), the assay with the lowest IC_{50} was chosen for further assay development. The procedure for competitive ELISA was essentially the same as that for the indirect ELISA described above except that dilutions of NPVs proteins were incubated overnight at room temperature with the antisera in PBSTA.

Cross-Reactivity (CR)

Data were obtained from standard inhibition curves for AcAaIT polyhedrin protein and other analytes by using the optimized ELISA system. The CR was calculated as the IC_{50} of AcAaIT divided by the IC_{50} of the analyte times 100.

DNA Analysis

Restriction Endonuclease Analysis

Viral DNAs were isolated according to [14]. Polyhedra were purified from infected larvae as described above. Virions were released from polyhedra by incubation with a DAS buffer at room temperature for 30 min. Undissolved polyhedra and heavy debris were removed by low speed centrifugation at 1,500g for 5 min. The supernatant was then centrifuged through a 36-56% (wt:wt) continuous sucrose gradient at 40,320g for 120 min. to pellet the ODVs. The bands containing ODVs were collected, washed twice with double-distilled water and finally re-suspended in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8) buffer. In order to release the viral DNAs, the gradient-purified ODVs were incubated in TE buffer containing 1 mg/ml proteinase K at 45-50°C for 2.5 h and then for an additional 0.5 h following the addition of 1% SDS at the same temperature. Viral DNAs were extracted once with TE buffer-saturated phenol and then several times with TE buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1) until the interface was completely clean. The aqueous phase containing the viral DNA was dialyzed at 4°C for 48 h against 3 or 4 changes of 10 mM TE buffer.

For restriction endonuclease (REN) analysis of AcAaIT, 1µg of DNA was incubated with 10 U of *Hin*dIII or *Bam*HI. Electrophoresis was performed using a horizontal 0.8% agarose gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0). The DNA fragments were visualized by staining with ethidium bromide.

Acute Oral and Injection Toxicity Studies of AcAaIT, AcMNPV and SlNPV in Albino Rats

Acute Oral and Injection Toxicity

The experimental and control group of rats consisted of three animals of each sex. A single dose of 1×10^8 PIBs in distilled water (maximum volume of 2 ml/100 gm body weight) was orally administrated to each animal using an ingestion needle. Rats in the untreated control group received 2 ml distilled water. Prior to exposure, the animals were fasted overnight, and prohibited from food for a further 3-4 h post administration.

The test procedures were carried out as described above, except that the test dose was administred intraperitoneal at a dose of 1×10^7 PIBs/animal in 0.9% NaCl saline (maximum volume of 1 ml/100 gm body weight).

The animals were observed for 21 days after treatment. Careful clinical examination and general observations of all animals were made daily. At the end of the observation period, the rats were sacrificed, and blood and histopathological investigations were carried out.

Blood Sampling

Blood from the sacrificed animals was divided into two samples. The first sample (about 0.5 ml) was collected in EDTA tubes, gently mixed, and used for haematological studies. The second sample (about 2.5 ml) was collected in clean centrifuge tubes and left at room temperature until clotting. After complete clot retraction, the samples were centrifuged at 5,000 rpm for 15 min at 4ºC, and the serum was separated and transferred to clean microfuge tubes for chemical analysis.

Effect on Blood Cells

The blood samples that were collected in EDTA tubes were subjected to red and white blood cell, and platelet quantification. Erythrocytes and platelets were counted

using an AO Bright line haemocytometer under light microscopy at 400x magnification. Blood samples for erythrocytes were diluted to 200 times original volume with physiological saline (0.9% NaCl solution) before counting, and blood samples for platelets were diluted to 20 times original volume with the same solution before counting [15]. Leukocytes were counted using an AO Bright line haemocytometer under light microscopy at 100x magnification after diluting the blood samples to 20 times original volume in a 1% acetic acid solution containing 1 mg of Leishman's stain [16].

Effect on Blood Serum Chemistry

The serum activity of acetylcholinesterase (AChE) [17], total protein [18], alkaline phosphatase [19], aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [20], glucose [21], urea [22], creatinin [23], and bilirubin [24] were estimated as described.

Electrophoretic Assay of Serum Proteins

SDS-PAGE was used to analyze serum protein levels in the albino rats following the various treatment regimes. SDS-PAGE was performed at room temperature in a vertical gel apparatus as described by [11]. The separating gel (10% acrylamide) contained 10 ml of acrylamide-bis (30%, 29.2 gm acrylamide and 0.8 gm bis-acrylamide); 7.5 ml of 2 M Tris-HCl, pH 8.8; 400 µl SDS (10%); 11.1 ml distilled water; 1.0 ml ammonium persulfate (APS 1.5%); and 15 µl TEMED. The stacking gel (3.5% acrylamide) contained 3.5 ml of acrylamide–bis (30%); 2.8 ml of 0.5 M Tris-HCl, pH 6.8; 300 µl SDS (10%); 17.7 ml distilled water; 1.0 ml APS (1.5%); and 25 µl TEMED. A tris-glycine buffer, pH 8.3, system containing 3.0 gm Tris; 14.4 gm glycine; 1.0 gm SDS; and diluted to 1.0 liter with distilled water was used as the electrode buffer in a slap plate electrophoretic unit (Marysol Gel Electrophoresis system). The gel was run for 5 h with a circulating current of 35 mA/plate using a Shandon Southern Power supply. The gel was stained overnight in the staining solution (2 gm Coomassie Brilliant Blue R-250, (0.2%); 300 ml ethanol (30%); 50 ml acetic acid (5%); and 650 ml distilled water. A destaining procedure was carried out to remove background staining by immersing the gels overnight in 96% acetic acid, ethanol, and distilled water (2:12:28, v:v:v).

The protein sample for loading was prepared by mixing 20 µl of serum with 40 µl sample buffer (1 gm SDS, 1.5 ml 2-mercaptoethanol, 5 ml glycerol, and 0.02 gm bromophenol blue dissolved in 50 ml Tris, pH 6.8). The loading sample was incubated at 90ºC for 5 min., then 25 µl was applied into each single slot of the slap gel. A mixture of standard proteins (phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme; 97, 66, 45, 31, 21, and 14 KDa, respectively) was used as molecular weight standards.

Histopathological Effect

Tissue specimens from stomach, intestine, liver, kidney, brain, spleen, and lung were obtained from male and female rats at 21 days post baculovirus treatment, and subjected to examination by light microscopy and photographed.

Effect of AcAaIT, AcMNPV, and SlNPV on Macrophage Phagocytosis in Fish

The fish (*T. nilotica*), each weighing approximately 10 gm, were divided into four groups of 10 fish per group. Three of the groups were treated with virus (AcAaIT, AcMNPV or SlNPV) that was incorporated into their standard food [25] at the rate of 1 x 10^9 PIBs/group for two days. The fourth group was reserved as a control. During the experimental period (30 days), the water was changed every two days.

Collection of Head Kidney Macrophages

At the end of the experimental period, the fish were taken from their containers and laid on their side. An incision was made from the anus to the pectoral fins, then perpendicularly to the top of the head using scissors. The viscera were pulled gently with forceps in order to get the head kidney. After removing the swim bladder, the head kidney was excised and placed in a petri dish with sterile Minimum Essential Medium (MEM) containing 10% fetal calf serum. All steps were carried out under aseptic conditions. Cell suspensions were prepared by teasing the tissue with a glass rod and passing them through a nylon sheath, then centrifuged at 800g for 10 min. The supernatant was discarded and the pellet was resuspended in 7 ml of sterile MEM.

Phagocytosis Assay

Phagocyte viability was evaluated by mixing 100 µl of the macrophage suspension with 100 µl of 0.4% trypan blue, and counting 100 cells under phase contrast microscopy [26]. A macrophage suspension in MEM (1 x 10^6 viable cells) was placed in a test tube containing formalin-killed bacteria (1 x 10^8 *Escherichia coli* in 0.5 ml). Phagocytosis was allowed to proceed at 10°C, and samples were taken at 30 min intervals. Phagocytosis was quantified by mixing 100 µl of the previous suspension with 100 µl of Wright's stain, and determining the proportion of macrophages that contained intracellular bacteria in a random count of 100 macrophages and expressed as percent phagocytosis. The results were expressed as the mean ± S.D.

Results

Analysis of Structural Proteins by SDS-PAGE

Third instar *S. littoralis* were inoculated by introducing them to cotton plants treated with AcAaIT at a concentration that was adjusted to resemble the

recommended field application rate (4 x 10^{12} PIB/feddan). Under the conditions tested, all of the exposed larvae became highly infected. No marked changes in the protein profiles of the field-produced AcAaIT (in larvae of *S. littoralis*) were found in comparison to control laboratory-produced AcAaIT, AcMNPV, or SlNPV by SDS-PAGE (Fig. 1) and ELISA (Table 2). Specifically, a single major protein band with an estimated molecular weight of 32 KDa was identified by SDS-PAGE from polyhedra isolated from these viruses (Fig. 1A). SDS-PAGE of ODV of these viruses, however, showed some minor differences (Fig. 1B).

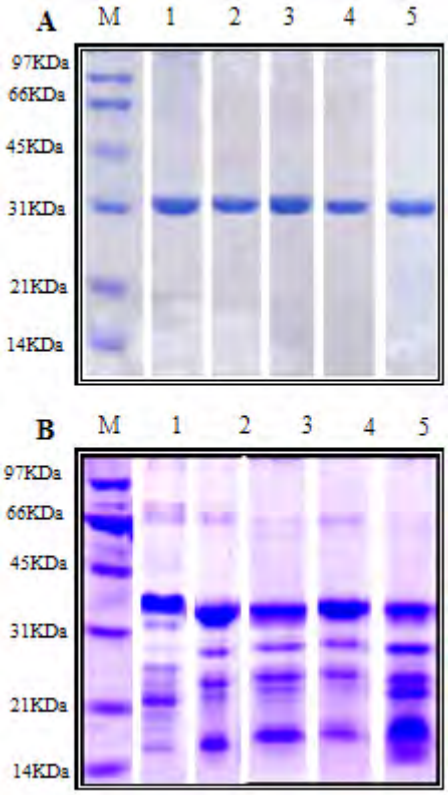

Figure 1: SDS-PAGE analyses of polyhedra (A) and ODVs (B) of AcAaIT (lanes 1), AcAaIT-laboratory (lanes 2), AcAaIT-field (lanes 3), AcMNPV (lanes 4), and SlNPV (lanes 5). The molecular weight of size standards (lanes M) are indicated to the left.

Enzyme-Linked Immunosorbent Assay for AcAaIT Polyhedrin Protein

Antisera against AcAaIT polyhedrin were raised in rabbits. The terminal bleeds from rabbits were screened against four coating antigens (i.e., polyhedrin from polyhedra of AcAaIT, AcAaIT-field, AcMNPV, and SlNPV) using a two-dimensional titration method with the coated antigen format.

The optimal concentrations of coating antigen, AcAaIT polyhedrin, and antisera were obtained by checkerboard titration assays (Table 1 and Fig. 2). The combination of coating antigen and antibody that resulted in the highest titer was selected for further development.

Table 1: Absorbance at 450 nm using different concentrations of antigen and different dilutions of serum antibody.

Antibody dilutions	*Antigen concentrations**					
	20 µg/ml	*10 µg/ml*	*5 µg/ml*	*2.5 µg/ml*	*1.25 µg/ml*	*0.0 µg/ml*
1:1000	0.427	0.417	0.416	0.414	0.399	0.075
1:2000	0.411	0.403	0.409	0.397	0.392	0.074
1:4000	0.386	0.391	0.381	0.373	0.37	0.071
1:8000	0.345	0.349	0.354	0.345	0.318	0.076
1:16000	0.279	0.295	0.31	0.299	0.262	0.073
1:32000	0.218	0.253	0.253	0.249	0.229	0.075
1:64000	0.173	0.198	0.21	0.203	0.175	0.078
0.0	0.075	0.071	0.079	0.076	0.071	0.074

*The antigen was AcAaIT polyhedrin.

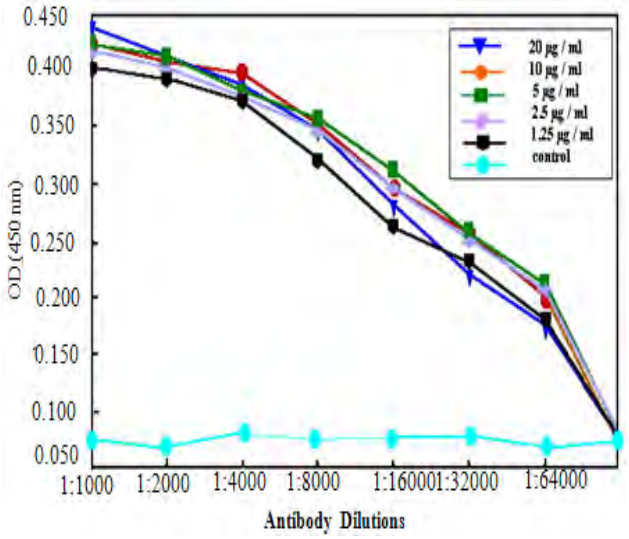

Figure 2: Two-dimentional titration of various dilutions of serum against AcAaIT polyhedrin with different concentrations (0.0, 1.25, 2.5, 5, 10 and 20 µg/ml) of AcAaIT polyhedrin as the coating antigen.

A competitive inhibition experiment was conducted in parallel to optimize antibody and coating antigen concentrations. Antibody dilution was 1:4,000 and the coating antigen concentration was 2.5 µg/ml. The lowest IC_{50} value was chosen as the optimal combination. The IC_{50} values for each combination ranged from 1.42 to 163 µg/ml (Table 2 and Fig 3). AcAaIT polyhedrin gave the lowest IC_{50} value, followed by polyhedrin from SlNPV, AcAaIT-field, and AcMNPV.

Table 2: Selected competitive ELISA screening data against AcAaIT. AcAaIT polyhedrin was used as the coating antigen.

Analytes	*Coating antigen µg/ml*	*Antiserum dilution*	*IC_{50} µg/ml*	*Absorbance*		*Slope*	*Intercept*
				min.	*max.*		
AcAaIT	2.5	1/4000	1.42	0.099	0.239	0.45	4.93
AcAaIT-field	2.5	1/4000	122.93	0.195	0.296	0.37	4.23
AcMNPV	2.5	1/4000	163.38	0.109	0.323	0.84	3.15
SlNPV	2.5	1/4000	35.19	0.15	0.282	0.35	4.46

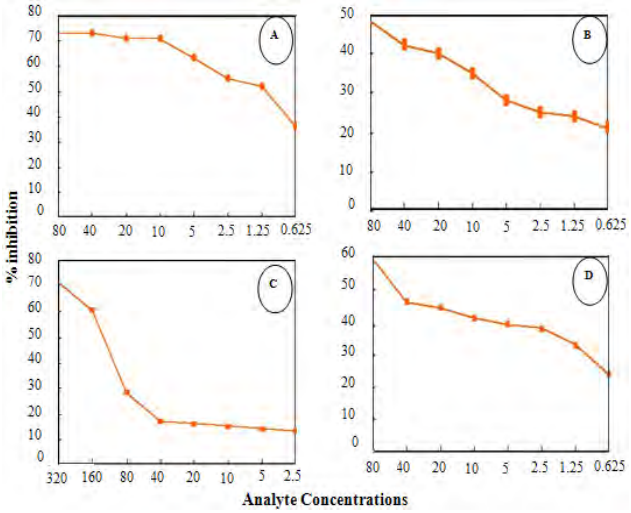

Figure 3: ELISA inhibition curves for 4 analytes: polyhedrin from AcAaIT (A), AcAaIT-field (B), AcMNPV (C), and SlNPV (D). Reagent concentrations: antiserum 1:4,000 (final dilution in wells); AcAaIT polyhedrin (2.5 µg/ml) was used as the coating antigen.

Table 3: Cross-reactivity of serum against AcAaIT polyhedrin

Origin of polyhedrin protein	*Cross-reactivity*(%)*
AcAaIT	100
AcAaIT-field	1.2
AcMNPV	0.87
SlNPV	4.0

*Cross-reactivity was calculated as (IC_{50} of AcAaIT/IC_{50} of analyte) x 100. AcAaIT polyhedrin was used as the coating antigen.

The cross-reactivity (CR) of the optimized ELISA system was low for wild type AcMNPV, AcAaIT-field, and wild type SlNPV (Table 3).

DNA Analysis

Agarose gel electrophoresis of restriction endonuclease *Hin*dIII or *Bam*HI) digested DNAs from AcAaIT; AcAaIT-laboratory, or AcAaIT-field indicated that these viruses were genetically similar. Specifically, there were no detectible differences in the digestion patterns of AcAaIT DNAs that were produced in laboratory or field infected larvae (Fig. 4).

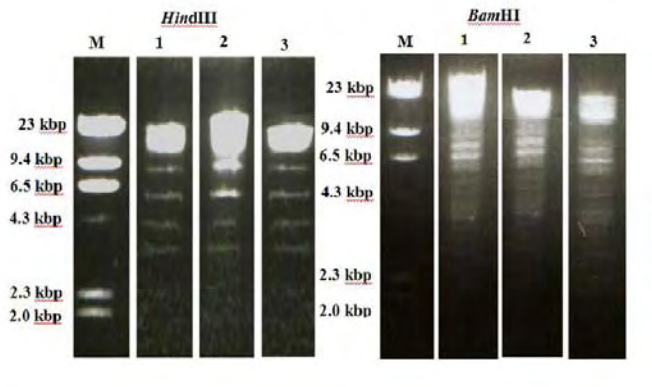

Figure 4: Restriction endonuclease profiles of DNAs isolated from AcAaIT (lanes 1), AcAaIT-laboratory (lanes 2), or AcAaIT-field (lanes 3) following treatment with *Hin*dIII or *Bam*HI and electrophoresis in a 0.8% agarose gel. The sizes of molecular weight standards of lambda phage DNA digested with *Hin*dIII (lanes M) are indicated to the left.

Acute Oral and Injection Toxicity of AcAaIT, AcMNPV and SlNPV in Albino Rats

Acute oral and intraperitoneal injection toxicity experiments were carried out on male and female albino rats treated with a single dose (oral: 1 x 10^8 PIBs/animal, intraperitoneal: 1 x 10^7 PIBs/animal) of AcAaIT, AcMNPV, or SlNPV. Blood samples and specimens from stomach, intestine, liver, kidney, brain, spleen, and lung were taken at 21 days post treatment and subjected to examination. Clinical observations during the experimental period showed that all rats were apparently healthy and showed a slight increase in the body weight.

Effect on Blood Cells

No marked differences were found in erythrocytes or platelet numbers in treated and untreated animals (Tables 4, 5, and 6). Leukocytes numbers increased by 65.3% and 79.8% in females treated with AcMNPV and SlNPV, respectively.

Table 4: Leucocyte numbers following oral or intraperitoneal injection treatment of rats with SlNPV, AcMNPV or AcAaIT.

Groups	*Oral*		*Injection*	
	Male	*Female*	*Male*	*Female*
Control	17.08±3.36*	12.00±2.54	16.92±3.08	10.57±2.74
SlNPV	15.17±2.47 (-11.2)**	15.43±3.8 (+28.6)	16.63±4.34 (-1.7)	19.00±1.53 (+79.8)
AcMNPV	13.82±3.91 (-19.1)	19.83±0.29 (+65.3)	16.80±4.2 (-0.7)	16.15±3.70 (+52.8)
AcAaIT	15.17±4.33 (-11.2)	14.03±2.53 (+16.9)	14.92±2.15 (-11.8)	14.48±2.98 (+37.1)

-Each rat was given a single oral dose (1 x 10^8 PIBs/rat) or injection (1 x 10^7 PIBs/rat). *Number of white blood cells/mm^3 (mean ± S.D.) x 1,000 at 21 days post treatment. **Values in parentheses are % differences [(treated – control) / control x 100].

Table 5: Erythrocyte numbers following oral or intraperitoneal injection treatment of rats with SlNPV, AcMNPVor AcAaIT.

Groups	*Oral*		*Injection*	
	Male	*Female*	*Male*	*Female*
Control	7.9±0.5*	10.5±0.9	7.8±1.7	9.6±1.3
SlNPV	9.4±1.04 (+18.2)**	10.8±1.2 (+2.4)	9.2±1.4 (+18.4)	7.2±1.3 (-24.7)
AcMNPV	8.5±1.1 (+7.5)	10.3±1.1 (-1.9)	7.2±1.4 (-7.3)	10.2±1.9 (+5.7)
AcAaIT	7.3±1.9 (-8.0)	10.1±0.7 (-4.0)	7.9±0.5 (+1.2)	10.3±3.8 (+7.0)

-Each rat was given a single oral dose (1 x 10^8 PIBs/rat) or injection (1 x 10^7 PIBs/rat). *Number of red blood cells/mm^3 (mean ± S.D.) x 10^6 at 21 days post treatment.
**Values in parentheses are % differences [(treated – control) / control x 100].

Table 6: Platelet numbers following oral or intraperitoneal injection treatment of rats with SlNPV, AcMNPV or AcAaIT.

Groups	*Oral*		*Injection*	
	Male	*Female*	*Male*	*Female*
Control	261.3±16.2*	310.7±64.3	280.7±70.5	263.7±41.7
SlNPV	219.7±28.4 (-15.9)**	281±45.4 (-9.5)	259±50.5 (-7.7)	260±18.02 (-1.4)
AcMNPV	283.7±11.6 (+8.5)	338.7±52.4 (+9.0)	303±43.3 (+8.0)	342±82.4 (+29.7)
AcAaIT	238±23.3 (-8.9)	330±63.5 (+6.2)	269.3±48.9 (-4.0)	276±58.8 (+4.7)

-Each rat was given a single oral dose (1 x 10^8 PIBs/rat) or injection (1 x 10^7 PIBs/rat).*Number of platelets/mm^3 (mean ± S.D.) x 1,000 at 21 days post treatment.**Values in parentheses are % differences [(treated – control) / control x 100].

Effect on Blood Serum Chemistry

The measurement of any single serum enzyme activity or component gives little information of the condition of an organ or organ system. Thus, a "chemistry panel" that includes several enzyme tests is generally used to rule out organ malfunction and to diagnose and monitor a variety of disorders [27]. The effects of baculovirus exposure on the serum levels of several key blood components are shown in Tables 7, 8, and 9. In terms of total serum protein levels (Table 7), oral exposure to SlNPV resulted in a 13.3% increase and 21.7% decrease in male and female rats, respectively. Male rats that were injected with SlNPV, AcMNPV or AcAaIT showed 13.9%, 23.5%, and 32.2% decreases in total serum protein levels, respectively, whereas, no dramatic differences were found in female rats. SDS-PAGE analysis of serum proteins appeared to be consistent with these results (Fig. 5).

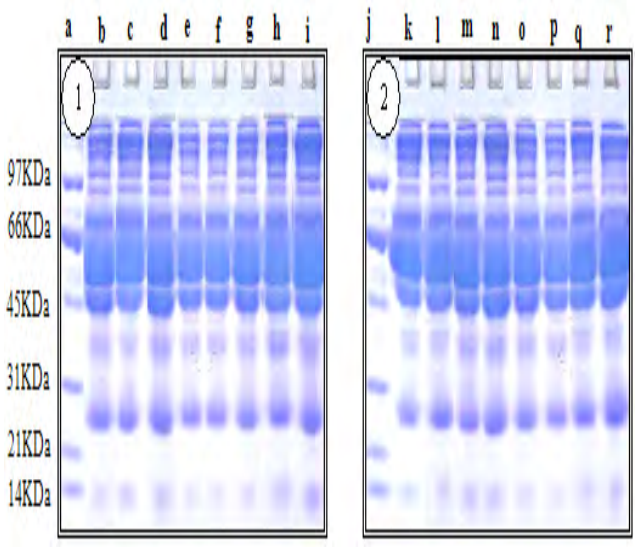

Figure 5: SDS-PAGE of serum proteins from rats at 21 days post oral treatment (panel 1) or intraperitoneal injection (panel 2) of baculovirus: control males (lanes b and k), control females (lanes c and l), SlNPV exposed males (lanes d and m), SlNPV exposed females (lanes e and n), AcMNPV exposed males (lanes f and o), AcMMPV exposed females (lanes g and p), AcAaIT exposed males (lanes h and q), AcAaIT exposed females (lanes i and r). The sizes of molecular weight standards (lanes a and j) are indicated to the left of panel 1.

In terms of serum glucose levels (Table 7), male rats that were orally exposed to the baculoviruses did not show any significant differences. In contrast, female rats showed 36.6% and 16.4% higher glucose levels when exposed orally to SlNPV or AcMNPV, respectively; and 40.2% lower levels when orally exposed to AcAaIT. Following intraperitoneal injection, male rats showed no significant differences in glucose levels; whereas female rats showed 27.3% and 33.9% decreases following injection with SlNPV and AcAaIT, respectively.

Table 7: Levels of total protein, glucose, and acetylcholinesterase in rat serum at 21 days after oral or intraperitoneal administration of SlNPV, AcMNPV or AcAaIT

Group		*Serum Level*					
		Oral			*Injection*		
		Total protein (gm/dl)	*Glucose (mg/dl)*	*Acetylcholinesterase (U/L)*	*Total protein (gm/dl)*	*Glucose (mg/dl)*	*Acetylcholinesterase (U/L)*
Control	Male	6.9 ± 0.6*	133.8 ± 12.0	5.1 ± 0.9	9.5 ± 1.5	147.9 ± 20.6	6.0 ± 0.6***
	Female	8.7 ± 1.4	138.3 ± 18.8	6.7 ± 0.9	8.1 ± 0.3	180.1 ± 35.0	5.6 ± 0.8
SlNPV	Male	7.8 ± 0.9 (+13.3)**	128.6 ± 12.3 (-3.9)	5.6 ± 0.9 (+10.5)	8.2 ± 0.5 (-13.9)	141.4 ± 37.0 (-4.4)	6.2 ± 0.6 (+3.8)
	Female	6.8 ± 0.1 (-21.7)	188.9 ± 11.5 (+36.6)	5.9 ± 1.0 (-12.5)	8.2 ± 0.8 (+1.1)	130.8 ± 8.7 (-27.3)	5.9 ± 0.9 (+6.2)
AcMNPV	Male	7.0 ± 0.8 (+1.7)	136.2 ± 4.8 (+1.8)	5.3 ± 0.4 (+3.5)	7.3 ± 1.0 (-23.5)	141.5 ± 25.7 (-4.3)	5.1 ± 0.5 (-15.2)
	Female	7.9 ± 0.6 (-9.5)	161.1 ± 33.4 (+16.4)	5.1 ± 1.0 (-24.5)	7.7 ± 0.4 (-4.2)	163.7 ± 24.1 (-9.1)	6.0 ± 0.9 (+7.6)
AcAaIT	Male	6.9 ± 0.4 (-0.4)	155.1 ± 9.0 (+0.2)	4.8 ± 0.9 (-4.9)	6.4 ± 0.6 (-32.2)	141.9 ± 22.9 (-4.1)	5.6 ± 1.1 (-5.3)
	Female	9.4 ± 1.04 (+8.2)	82.7 ± 12.7 (-40.2)	5.7 ± 0.7 (-14.7)	8.7 ± 1.8 (+7.8)	119.1 ± 12.8 (-33.9)	5.0 ± 1.0 (-10.7)

Rats were given a single oral dose ($1x10^8$PIB/rat) or injection ($1x10^7$PIB/rat).
*Values are the mean ± S.D. of 3 rats x 1 replicate each, 21 days post treatment.
**Values in parentheses are % differences [(treated – control) x100/ control].
***Values of Acetylcholinesterase in thousand.

Table 8: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin in rat serum at 21 days after oral or intraperitoneal administration of SlNPV, AcMNPV or AcAaIT

Groups		*Serum Level*							
		Oral				*Injection*			
		ALT (U/L)	*AST (U/L)*	*ALP (U/L)*	*Bilirubin (mg/dl)*	*ALT (U/L)*	*AST (U/L)*	*ALP (U/L)*	*Bilirubin (mg/dl)*
Control	Male	12.2 ± 2.1*	31.3 ± 1.3	105.4 ± 8.3	0.31 ± 0.06	10.0 ± 0.5	30.8 ± 1.8	72.7 ± 13.7	0.33 ± 0.07
	Female	8.0 ± 1.6	24.8 ± 3.3	81.8 ± 5.5	0.21 ± 0.04	8.8 ± 2.4	24.0 ± 1.3	76.4 ± 9.4	0.20 ± 0.03
SlNPV	Male	10.0 ± 1.3 (-18.1)**	25.8 ± 0.8 (-17.6)	67.3 ± 8.3 (-36.2)	0.34 ± 0.07 (+8.04)	14.4 ± 1.7 (+44.0)	25.3 ± 1.4 (-17.8)	70.9 ± 10.9 (-2.5)	0.38 ± 0.03 (+12.9)
	Female	6.9 ± 0.6 (-13.3)	18.7 ± 3.8 (-24.8)	72.7 ± 13.7 (-11.1)	0.28 ± 0.02 (+30.5)	7.2 ± 1.9 (-18.9)	19.8 ± 1.4 (-17.4)	61.8 ± 8.3 (-19.0)	0.36 ± 0.04 (+81.8)
AcMNPV	Male	10.1 ± 1.3 (-17.0)	26.2 ± 1.04 (-16.5)	87.3 ± 5.5 (-17.2)	0.21 ± 0.04 (-34.5)	10.9 ± 1.1 (+9.3)	27.2 ± 2.8 (-11.9)	58.2 ± 8.3 (-20.0)	0.27 ± 0.05 (-18.3)
	Female	6.7 ± 0.5 (-15.8)	18.2 ± 1.5 (-26.8)	74.5 ± 11.4 (-8.9)	0.20 ± 0.04 (-1.7)	6.5 ± 0.8 (-26.4)	20.3 ± 2.8 (-15.3)	69.1 ± 8.3 (-9.5)	0.20 ± 0.03 (0.00)
AcAaIT	Male	8.1 ± 1.6 (-33.2)	17.7 ± 0.8 (-43.6)	78.2 ± 11.4 (-25.8)	0.29 ± 0.03 (-8.04)	8.6 ± 0.9 (-14.3)	18.0 ± 1.0 (-41.6)	65.4 ± 10.9 (-10.0)	0.40 ± 0.04 (+19.4)
	Female	7.4 ± 1.3 (-7.1)	21.7 ± 3.7 (-12.8)	83.6 ± 11.4 (+2.2)	0.13 ± 0.01 (-37.3)	13.8 ± 0.8 (+56.6)	17.7 ± 3.8 (-26.4)	70.9 ± 9.4 (-7.1)	0.18 ± 0.01 (-7.3)

Rats were given a single oral dose (1×10^8 PIBs/rat) or injection (1×10^7 PIBs/rat)
*Values are the mean ± S.D. of the values from 3 rats at 21 days post treatment.
**Values in parentheses are % differences [(treated – control)/control x 100].

Table 9: Levels creatinin and urea in rat serum at 21 days after oral or intraperitoneal administration of SlNPV, AcMNPV or AcAaIT

Groups		*Serum Level*			
		Oral		*Injection*	
		Creatinin (mg/dl)	*Urea (mg /dl)*	*Creatinin (mg/dl)*	*Urea (mg /dl)*
Control	Male	3.63 ± 0.41*	43.7 ± 3.5	3.17 ± 0.13	43.0 ± 4.5
	Female	2.15 ± 0.07	39.1 ± 2.7	3.82 ± 0.13	45.9 ± 9.2
SlNPV	Male	3.64 ± 0.31 (+0.4)**	44.2 ± 2.2 (+1.1)	3.36 ± 0.22 (+6.1)	55.8 ± 4.8 (+30.0)
	Female	3.04 ± 0.10 (+41.4)	38.2 ± 7.3 (-2.2)	2.94 ± 0.30 (-23.1)	42.6 ± 3.0 (-7.2)
AcMNPV	Male	2.84 ± 0.03 (-21.6)	48.3 ± 10.1 (+10.5)	3.22 ± 0.01 (+1.5)	45.7 ± 8.2 (+6.3)
	Female	2.70 ± 0.11 (+25.7)	43.5 ± 12.4 (+11.3)	2.59 ± 0.12 (-32.2)	46.4 ± 5.9 (+1.03)
AcAaIT	Male	2.53 ± 0.05 (-30.2)	42.7 ± 5.0 (-2.2)	3.09 ± 0.03 (-2.3)	41.7 ± 4.3 (-2.9)
	Female	3.07 ± 0.14 (+42.9)	40.2 ± 8.9 (+2.8)	2.61 ± 0.17 (-31.8)	42.2 ± 2.6 (-8.1)

Rats were given a single oral dose (1 x 10^8 PIBs/rat) or injection (1 x 10^7 PIBs/rat)
*Values are the mean ± S.D. of the values from 3 rats at 21 days post treatment.
**Values in parentheses are % differences [(treated – control)/control x 100].

In terms of serum acetylcholinesterase (AChE) activity (Table 7), male rats that were orally exposed to the baculoviruses did not show any significant differences. In contrast, female rats showed 12.5%, 24.5% and 14.7% lower AChE activities following oral exposure to SlNPV, AcMNPV, or AcAaIT, respectively. Male rats showed 15.2% lower AchE activity following intraperitoneal injection of AcMNPV, but no significant differences following injection with SlNPV or AcAaIT. No dramatic differences were found in AChE activities in female rats following baculovirus injection.

Serum activities/levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin (Table 8) are biomarkers for liver function. Following oral exposure with SlNPV, AcMNPV or AcAaIT, there appeared to be a general decline in most of these activities. Bilirubin levels, however, increased (30.5%) in rats that were orally exposed to SlNPV. Following interperitoneal injection, ALT activity increased in male SlNPV (44.0%) or AcMNPV (9.3%) injected rats, and female AcAaIT (56.6%) injected rats; whereas ALT activity decreased in female SlNPV- or AcMNPV-injected, and male AcAaIT-injected, rats. AST and ALP activities generally decreased (e.g., a 41.6% decrease in AST activity in male rats injected with AcAaIT). Bilirubin levels increased in SlNPV-injected rats (up to 81.8%), decreased in male rats injected with AcMNPV, and increased in male rats injected with AcAaIT.

Serum levels of creatinin and urea (Table 9) are biomarkers for kidney function. No changes in creatinin levels were found the following oral exposure of male rats to SlNPV; however, creatinin levels decreased (21.6% and 30.2%) in male rats that were orally exposed to AcMNPV or AcAaIT, respectively. In contrast, creatinin levels increased following the oral exposure of female rats to SlNPV, AcMNPV, or AcAaIT (41.4%, 25.7%, and 42.9%, respecitively). Following interperitoneal injection or SlNPV, AcMNPV, or AcAaIT, there were no major changes in creatinin levels in male rats; however, creatinin levels decreased in female rats (23.1%, 32.2%, and 31.8%, respectively). Following oral exposure to AcMNPV, urea levels increased in male and female rats. In contrast urea levels were unchanged in male and female rats that were orally exposed to SlNPV or AcAaIT. Following interperitoneal injection of SlNPV, creatinin levels increased (30.0%) in male rats. There were no dramatic differences in creatinin levels in AcMNPV- or AcAaIT-injected male or female rats.

Histopathological Effects

Tissues from stomach, intestine, liver, kidney, brain, spleen and lung were dissected from male and female rats that were orally exposed or intraperitoneally injected with SlNPV, AcMNPV or AcAaIT. The tissues were prepared at 21 days posttreatment for histochemical examination by light microscopy. The histopathological investigations

(Figs. 6-12) revealed no gross pathological differences between the various baculoviral treatments.

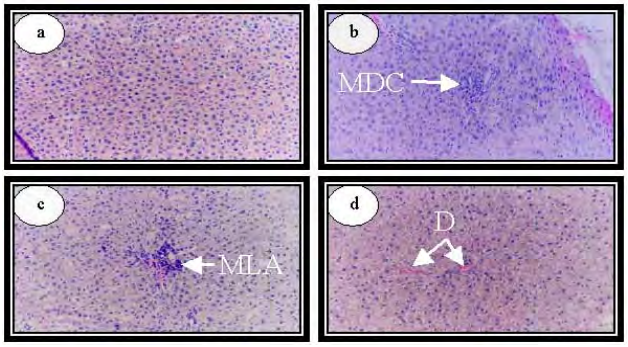

Figure 6: Histopathology of liver from female rats treated with saline (a) showing normal hepatic parenchyma; AcAaIT (b) showing mild reversible degenerative changes (MDC) in a few hepatocytes; AcMNPV (c) showing mild leukocyte aggregation (MLA) in the portal area; and SlNPV (d) showing vascular and hydropic degeneration (D) of some hepatocytes (H&E x300).

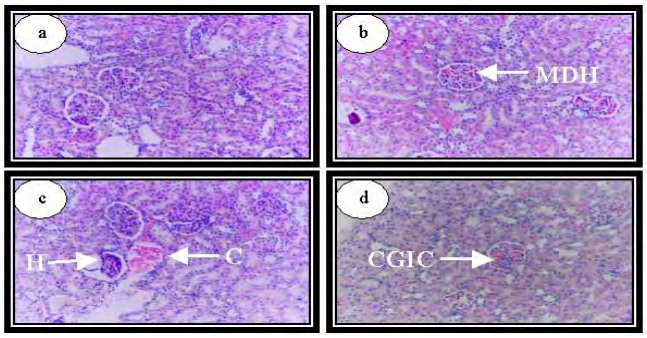

Figure 7: Histopathology of kidney from female rats treated with saline (a) showing normal renal tissue structures; AcAaIT (b) showing mild dilation and hyperemia (MDH) of glomerular capillaries; AcMNPV (c) showing congested (C) renal blood vessels and hypercellularly (H) of some glomeruli; and SlNPV (d) showing congested glomeruli and interregnal capillaries (CGIC) (H&E x300).

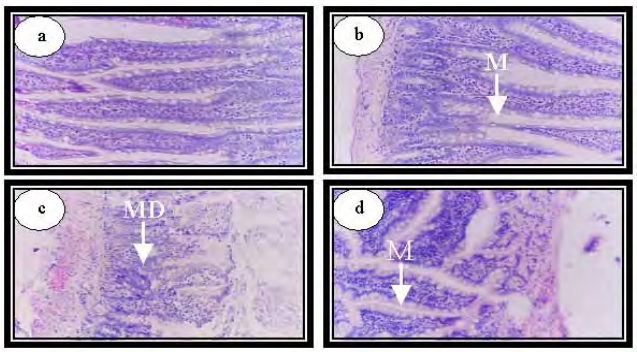

Figure 8: Histopathology of intestine from female rats treated with saline (a) showing showing apparently normal intestinal mucosa; AcAaIT (b) showing metaplasia (M) to goblet cells in the villus epithelium; AcMNPV (c) showing mucinous degeneration (MD); and SlNPV (d) showing mild mucus and metaplasia (M) of intestinal epithelium to global cells (H&E x300).

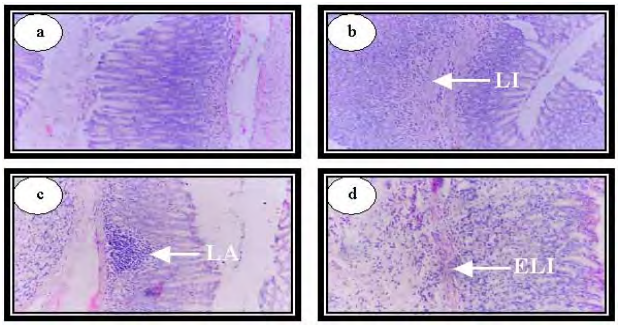

Figure 9: Histopathology of stomach from female rats treated with saline (a) showing normal gastric mucosa; AcAaIT (b) showing leukocytic infiltration (LI) in mucosa and submucosa; AcMNPV (c) showing lymphocytic aggregation (LA) in gastric mucosa; and SlNPV (d) showing edema and leukocytic infiltration (ELI) in gastric submucosa (H&E s300).

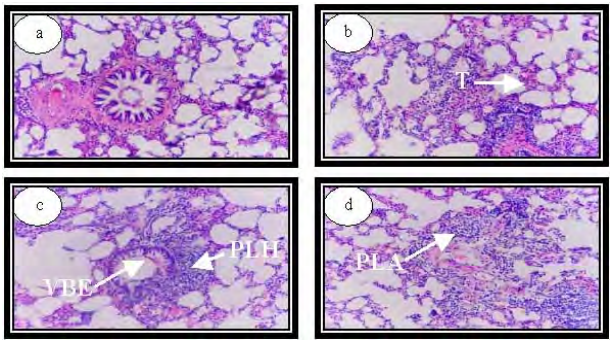

Figure 10: Histopathology of lung from female rats treated with saline (a) showing normal pulmonary tissue; AcAaIT (b) showing mild thickening (T) of some interaveolar septa; AcMNPV (c) showing vaculated bronchialar epithelium (VBE) and peribronchial lymphoid hyperplasia (PLH); and SlNPV (d) showing perivascular lymphoid aggregates (PLA) (H&E x300).

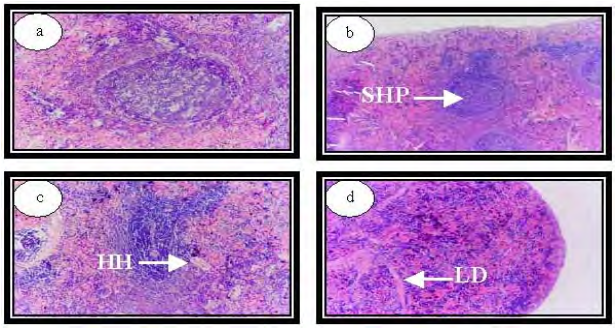

Figure 11: Histopathology of spleen from female rats treated with saline (a) showing normal splenic tissue; AcAaIT (b) showing scattered hemosidrin pigment (SHP) in the splenic red pulps; AcMNPV (c) showing mild hyperplasia of white pulps and hemosidrosis (HH); and SlNPV (d) showing mild lymphoid depletion (LD) (H&E x300).

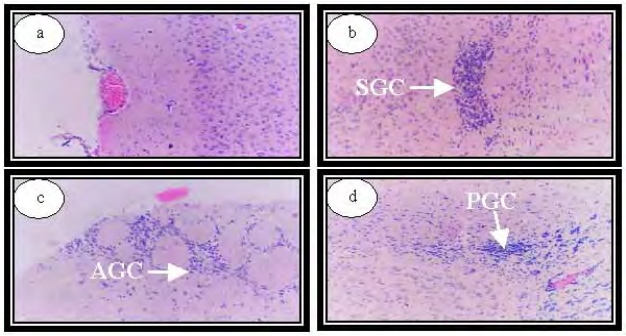

Figure 12: Histopathology of brain from female rats treated with saline (a) showing normal brain tissue; AcAait (b) showing scatterd glial cells (SGC) within the brain tissue; AcMNPV (c) showing aggregation of glial cells (AGC) between nerve fibers; and SlNPV (d) showing proliferation of glial cells (PGC) in the cerebral cortex (H&E x300).

Effect of the SlNPV, AcMNPV and AcAaIT on Macrophage Phagocytosis in Fish

Phagocytic activity of macrophages was determined following exposure of fish (*T. nilotica*) to SlNPV, AcMNPV or AcAaIT (Table 10). The fish were exposed by incorporating the virus (1 x 10^9 PIBs/group) into the standard diet. No mortality was induced in treated and untreated fish during the experimental period (28 days after initial treatment). Phagocytic activity increased during the time of the assay, reaching maximum values by 180 min. for both control and treated fish. However, in comparison to control macrophages, there was a general decrease in phagocytic activity in all of the baculovirus-exposed fish. This trend was most dramatic prior to the first 60 min. of the assay. In comparison to the control, the percentage reduction in phagocytic activities at 30 min. and 180 min. were 17.7% and 5.9% (SlNPV exposed); 19.5% and 1.7% (AcMNPV exposed); and 24.6% and 1.4% (AcAaIT exposed), respectively.

Table 10: Phagocytic activity of head kidney cells from *T. nilotica* exposed to SlNPV, AcMNPV or AcAaIT.

Treat-ment	*Phagocytic activity (mean ± S.D.)*					
	30 min	*60 min*	*90 min*	*120 min*	*150 min*	*180 min*
Control	73.3±5.8	78.3±7.6	86.7±7.6	93.3±1.5	93.3±1.5	94.3±2.5
SlNPV	60.3±9.0 (-17.7)*	71.0±5.3 (-9.3)	80.7±5.1 (-6.9)	84.0±7.9 (-9.9)	86.7±5.8 (-7.1)	88.7±7.8 (-5.9)
AcMN-PV	59.0±3.6 (-19.5)	67.0±6.1 (-14.4)	75.0±5.0 (-13.5)	83.3±5.8 (-10.7)	92.3±3.1 (-1.1)	92.7±3.5 (-1.7)
AcAaIT	55.3±5.0 (-24.6)	66.7±5.8 (-14.8)	80.7±7.0 (-6.9)	88.0±4.4 (-5.7)	90.3±0.6 (-3.2)	93.0±1.7 (-1.4)

*Values in parentheses are % differences [(treated – control)/control x 100].

Discussion

Increased knowledge regarding the undesired side effects of chemical insecticides such as the development of insecticide resistance and elimination of non-target or beneficial insects within an ecosystem, has resulted in intensified interest in the search for safer agents and methods for plant protection. The use of biological insecticides, either alone or in combination with other agents and methods in an integrated pest management system in crops with several pests, has been studied mainly with respect to efficacy against the target pest.

Viruses comprise an important class of insect pathogenic organism. Baculoviruses have long been considered as potential biopesticides due to their narrow host specificity, environmental friendliness, and other advantageous characteristics. Natural or wild type baculoviruses, however, have long killing times. Recombinant DNA technology has been used to overcome this and other potential drawbacks that have been associated with the large-scale use of baculovirus-based biopesticides [1, 2]. To date, several pesticidal genes have been inserted into the genomes of AcMNPV and other baculoviruses that infect pest insect species. The efficacy of these constructs has been evaluated under laboratory and field conditions [1-6]. A major strategy for improving the insecticidal efficacy of the baculovirus has been the insertion of insect-selective neurotoxin genes into the baculovirus genome. For example, the recombinant AcAaIT was developed by inserting the *aait* neurotoxin gene, which was isolated from the North African scorpion *A. australis*, into the genome of AcMNPV [5, 6].

The pathogenicity and virulence of AcAaIT, AcMNPV, and SlNPV have been studied in both field and laboratory colonies of the Egyptian cotton worm *S. littoralis* [28]. In this species, pathogenicity and virulence of SlNPV at LC_{50}, LC_{90}, and LT_{50} levels appears to be the highest, followed by AcAaIT and AcMNPV. In the present study, an artificial inoculation of third instar larvae of *S. littoralis* was carried out by introducing healthy larvae on cotton plants (under field conditions) treated with AcAaIT. All of the exposed larvae became highly infected and died in these experiments.

The potential of AcAaIT, AcMNPV, and SlNPV to induce adverse effects in a mammal and fish were determined in this study. We hope that our findings will be useful in providing a primary database for the establishment of future environmental risk assessment protocols and guidelines with baculovirus pesticides under the local environment. This will enable the competent authorities to make informed science-based decisions regarding genetically modified organisms and achieve biosafety protocols to protect man and biological diversity from the potential risks of modern biotechnology pest control agents.

Changes in the genomic or proteomic characteristics of a released baculovirus are indicators of its stability in the field. Agarose gel electrophoresis of DNAs from AcAaIT, AcAaIT-laboratory, and AcAaIT-field indicated

that the tested viruses were genetically similar, i.e., genomic differences were not found following passage in the field. Differences in viral structural proteins (e.g., polyhedrin and ODV-derived proteins) were also not detected in field-propagated virus by SDS-PAGE and ELISA analyses. Our results were consistent with previous studies [29-32].

Male and female albino rats that were orally exposed or intraperitoneally injected with AcAaIT, AcMNPV or SlNPV showed no mortality over a 21 day-long observation period. Visual observation of the rats during this period showed no indications of toxicity. No or slight indications of toxicity were noted by standard hematological and clinical biochemical assays. The apparent toxicity of injected viruses to oral exposure was assumed to be related to a general foreign body response. Our findings are in general agreement with previous studies concerning the effect of NPV exposure in non insect cells and tissues NPVs [33-40]. Additionally, there was no correlation between any effects induced by the wild type or recombinant baculoviruses in rats or fish.

Baculoviruses have been found only in invertebrates, no member of this family is known to infect vertebrates or higher plants [41]. Although there is a wealth of information regarding the relative safety of baculovirus-based biopesticides, major questions and hurdles still face the applied entomovirologist that is attempting to set-up a control program in an agro-ecosystem using existing baculovirus technology [42-44]. Some of the questions that need to be addressed in a particular ecosytem may include the ecology of the host insect, its horizontal and vertical distributions, the application of other pesticides through the host plant developmental stages, the persistence of endogenous viral types which could recombine with the applied biopesticide, and impact on nontarget organisms and beneficial insects [45, 46].

In conclusion, our findings are in agreement with previous studies that indicate that natural and recombinant baculoviruses provide no increased threat to mammals, fish, or other non-target organisms. We believe that baculovirus-based biopesticides are safe and effective and ready for immediate use in the field.

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