Insecticidal Properties and Microbial Contaminants in a *Spodoptera exigua* Multiple Nucleopolyhedrovirus (Baculoviridae) Formulation Stored at Different Temperatures

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ABSTRACT The *Spodoptera exigua* (Hübner) multiple nucleopolyhedrovirus (SeMNPV) is currently being tested as a biological insecticide for use in greenhouse crops in southern Spain. We performed a study in which semipurified SeMNPV occlusion bodies (OBs) were formulated in phosphate-buffered saline, pH 6.5, with 5% (vol:vol) glycerol and 0.15% (wt:vol) sorbic acid, and they were stored at −20, 4, or 25°C during 18 mo. Initial aerobic counts (±SE) averaged 1.4 (±0.17) × 10^7 colony-forming units/ml after 17-h incubation at 37°C. Aerobic counts of microorganisms that contaminated OB formulations stored at 25°C decreased markedly over the period of the study, whereas only small decreases were observed in counts from OBs stored at 4 or −20°C. The principal microbial contaminants of OB suspensions were *Enterococcus* spp., Enterobacteriaceae, and yeasts. Potential human pathogens (*Salmonella*, *Shigella*, and *Vibrio* species) were not detected, and populations of *Staphylococcus aureus* and *Bacillus cereus* were extremely low. Compared with newly formulated OBs, the estimated LD_{50} values of OBs stored at 25°C increased by >16,666-fold over the 18 mo of storage, whereas LD_{50} values were not greatly affected by storage at 4 or −20°C. Significant changes over time in OB concentrations were only observed in the 25°C treatment. Complete degradation of viral DNA was observed at 25°C but not in refrigerated or frozen OBs. We conclude that OB formulation with bacteriostatic or antioxidant additives, together with storage and distribution in refrigerated conditions, will likely result in an SeMNPV biopesticide shelf life that exceeds 18 mo.

KEY WORDS nucleopolyhedrovirus, *Spodoptera exigua*, microbial contaminants, storage

Baculoviruses are promising biopesticides and several have been commercialized for control of lepidopterous pests of crops or forests, in both developing and developed countries (Moscardi 1999). The multiple nucleopolyhedrovirus (SeMNPV, Baculoviridae) of the beet armyworm, *Spodoptera exigua* (Hübner), forms the basis for a number of efficient biopesticide products that are marketed in the United States, some European countries and many parts of southern Asia (Hunter-Fujita et al. 1998). Although in vitro production of baculovirus insecticides has attracted considerable attention and research effort, it is still not commercially viable (Szewczyk et al. 2006). Consequently, all baculovirus insecticides marketed to date are the result of in vivo production by using larvae of the original host or a closely related species.

Production of these viruses in living insects results in microbial contamination of viral occlusion bodies (OBs) that are released from the corpses of insects that die from virus infection. Such contaminants may affect the physical stability and insecticidal properties of formulated products or may present human health risks if known human pathogens are present (Podgwaite et al. 1983, Grzywacz et al. 1997). Accordingly, the identity and concentration of microbial contaminants is the subject of scrutiny by pesticide registration bodies.

Previous studies have examined the effectiveness of methods for reducing the quantity of microbial contaminants before formulation, either by centrifugation and chemical decontamination (Krieg et al. 1979), or by harvesting the OBs at different intervals postinfection (Smits and Vlak 1988, Cherry et al. 1997, Grzywacz et al. 1998). However, these processes can be inefficient (Grzywacz et al. 1997) and expensive in terms of time, labor, and equipment, so that procedures that avoid the need for purification steps improve both the speed and costs involved in baculovirus production (Shapiro 1982, Burges and Jones 1998). The magnitude of changes in the abundance of contaminant microorganisms in OB formulations during storage, and the relationship between microbial contaminants and the insecticidal activity of OBs, have not been addressed in detail.

A Spanish isolate of SeMNPV is currently being tested for control of *S. exigua* in greenhouse crops in Almería, southern Spain (Lasa et al. 2007a, 2007b). An

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experimental laboratory dedicated to production of SeMNPV in the Universidad Pública de Navarra, Pamplona, Spain, is currently producing $\approx 3 \times 10^{14}$ OBs annually, which is sufficient to treat >600 ha of greenhouse crops. This system of OB production and formulation forms the basis for a commercial-scale production plant that is currently being constructed by a grower's cooperative organization in Almería.

Information on the identity, abundance and influence of microbial contaminants on insecticidal activity is relevant to the registration of a biological insecticide based on SeMNPV. As the abundance of microorganisms may change during storage, we examined changes in microbial load in a simple OB formulation stored at different temperatures over an 18-mo period, and the influence of storage temperature on insecticidal activity.

Materials and Methods

Insects and Virus Production. Mass production of *S. exigua* larvae was performed using a laboratory colony maintained on semisynthetic diet in the insectary at the Universidad Pública de Navarra, Pamplona, Spain. Semisynthetic diet, adapted from Hoffman's tobacco hornworm diet (Hunter-Fujita et al. 1998), composed of a mixture of 850 ml of water, 72 g of wheat germ, 33 g of casein, 29.3 g of sucrose, 14.3 g of dried brewer's yeast, 9.4 g of Wesson's salts, 1.5 g of sorbic acid, 0.94 g of cholesterol, 0.94 g of sodium methyl *p*-hydroxybenzoate, 18.8 g of industrial agar, and 1.87 ml of linseed oil. These compounds were mixed and autoclaved and allowed to cool to 55–60°C, whereupon the following ingredients were added: 0.25 g of streptomycin, 1.65 g of tetracycline, 3.6 g of ascorbic acid, 0.1 g of vitamin mixture [30% (wt:wt) nicotinic acid, 30% calcium pantothenate, 7.5% thiamine, 15% riboflavin, 7.5% folic acid, and 7.5% pyridoxine hydrochloride], and 0.94 g of choline chloride. The colony was maintained using clean practices to minimize contamination, including the use of disposable paper bags for oviposition and sterile disposable plastic boxes for rearing larvae. Eggs and pupae were routinely decontaminated by immersion in 0.01 or 0.02% (wt:vol) sodium hypochlorite solution for 5–10 min, respectively. A solution of 0.4% sodium hypochlorite was used for routine decontamination of work surfaces and UV lamps were left on at night in the work area to reduce contamination.

Laboratory production of a Spanish isolate of SeMNPV (Caballero et al. 1992), involved inoculation of groups of 100 *S. exigua* fifth instars. These larvae were individualized in ventilated rectangular (200- by 300- by 60-mm) plastic boxes and allowed to feed on a slice of diet (100 by 50 by 2 mm) that had been previously sprayed on the upper and lower surfaces with a 95% lethal concentration of OBs, comprising 1.5×10^8 OBs, in a volume of 1.5 ml of sterile water, using an artist's air-brush (Paasche VL, Paasche Airbrush Co., Chicago, IL). The contaminated diet was placed on a sterile plastic mesh platform, with 10-mm rectangular holes, to allow feeding from beneath.

Larvae were held in a dark climate-controlled room at 25 ± 1°C and 60 ± 10% RH. Two days after inoculation, a new piece of untreated diet was placed in each box of larvae. Boxes were checked daily for virus mortality from 5 to 8 d postinoculation. Dead and moribund larvae were carefully collected using entomological tweezers and stored in sterile 250-ml glass jars at −20°C. On the eighth day, when virtually all larvae had died, each box was placed in a −20°C freezer to avoid loss of OBs from lysis of the insect tegument, and the remaining larvae were collected by scraping the inside of the box with a spatula.

For formulation, infected material stored in glass jars was allowed to thaw overnight, triturated in a blender and homogenized in phosphate-buffered saline (PBS), previously adjusted to pH 6.5 by using hydrochloric acid, and mixed with 0.15% (wt:vol) sorbic acid and 5% (vol:vol) glycerol. Sorbic acid was added to reduce the growth of microbial contaminants and the glycerol to increase the viscosity of the suspension. The resulting slurry was filtered through 200- and 40-μm pore size iron meshes to remove debris.

Aerobic Counts on Microbial Contaminants. An 800-ml sample collected in October 2004, during the preparation of 30 liters of OB formulation, was placed in a sterile glass flask and found to contain 6.85×10^{11} OB/liter by counting duplicate samples (two counts per sample) in a Neubauer hemocytomer under a phase contrast microscope at 400×.

Counts of aerobic microbial loads were conducted using LB agar petri plates inoculated with 100 μl of serially diluted OB suspensions. Plates were incubated at 37 ± 0.5°C for 17 h, examined for the presence of colony-forming units (CFU), and then reincubated at 28 ± 0.5°C and checked at 48 and 72 h for the development of new colonies. The SeMNPV sample was divided in volumes of 50 ml, placed in dark plastic screw-top bottles of 100-ml capacity and stored at −20 and 4°C in a laboratory freezer and fridge, respectively, or 25°C in a laboratory incubator, over a period of 18 mo. Three replicate bottles were stored at each temperature. CFU counts were performed at 3-mo intervals during the 18-mo storage period. The frozen virus stock was thawed each 3 mo, subjected to analysis, and refrozen. CFU counts were corrected by a factor of 1.37 to correspond with the concentration of the experimental formulation supplied to greenhouse growers for testing, that contained 5×10^8 OB/ml. CFU counts were normalized by log transformation and subjected to repeated measures analysis of variance (ANOVA) in SPSS version 12.0 (SPSS Inc., Chicago, IL).

Microbiological Analysis. After 18-mo storage, 35 ml of each sample batch was sent overnight to a microbiological analysis laboratory (Silliker Ibérica S.A., Barcelona, Spain), which conducted tests for the presence of human pathogens: *Vibrio* spp. (ISO 8914) *Shigella* spp., and *Salmonella* spp. (NF V08-052), and counts of total aerobic (NF V08-051) and anaerobic microorganisms (Pascual 1982), total Enterobacteriaceae (ISO 21528-2), *S. aureus* (ISO 6888), *Enterococcus* D Lancefield (ISO 7899), *Bacillus cereus* (ISO

7932), and yeast and mold (ISO 7954). Results were compared with a newly prepared batch of formulated OBs that had been adjusted to a similar concentration of 6.85×10^{11} OB/liter and placed in three replicate dark plastic bottles and sent for analysis together with three replicates of each of the stored samples. The results of the laboratory analyses were also corrected by a factor of 1.37 to account for concentration differences in the analyzed material and that supplied to growers for testing.

Physical Stability of OBs. After being thoroughly shaken, two 100-μl samples were taken from each bottle of formulated OB suspension to determine the concentration of OBs at 0, 6, 12, and 18 mo of storage. Each sample was diluted 100-fold and counted twice using a Neubauer improved chamber under a phase contrast microscope at 400×. Counts of OB concentration were subjected to repeated measures ANOVA in SPSS version 12.0 with storage period as the within-subject factor with three levels corresponding to 6, 12, and 18 mo.

Bioassays on Insecticidal Activity. The insecticidal activity of formulated batches of OBs was evaluated after 0, 6, 12, and 18 mo of storage. Bioassays were performed in second instars of *S. exigua* inoculated by the droplet feeding technique (Hughes et al. 1986). Groups of 30 recently molted larvae were allowed to feed on droplets of 10% (wt:vol) sucrose, 0.001 (vol:vol) fluorella blue food dye and one of a range of five concentrations of OBs of between 2.8×10^3 and 2.3×10^5 OB/ml, estimated to result in mortalities between 10 and 90%. In the case of suspensions stored at 25°C during 12 or 18 mo, concentrations in the range $2.1 \times 10^5 - 5 \times 10^7$ OB/ml were used. Twenty five larvae that had ingested the OB suspension within 10 min were placed individually into cells of a 25-cell tissue culture plate containing diet and incubated at 25 ± 2°C. Mortality was noted at 5 d postinoculation. Control larvae were treated identically but fed on a solution of sucrose and food dye alone. The bioassay was performed separately for each repetition. Results were subjected to logit regression with the Generalized Linear Interactive Modeling (GLIM) program (Numerical Algorithms Group 1993). A binomial error distribution was specified and minor overdispersion in the mortality results was taken into account by scaling the error distribution. Pathogenicity was expressed as the 50% lethal dose (LD_{50}) based on an average ingested volume of 0.33 μl per larva in this instar (Chaufaux and Ferron 1986, Muñoz et al. 1997).

Viral DNA and Restriction Endonuclease Analysis. A 2-ml volume of each OB sample was washed twice with 0.1% sodium dodecyl sulfate (SDS) and suspended in bidistilled water. Virions were released from the OBs by incubation in a solution of 1.25% SDS and 0.125 M Na_2CO_3. DNA was extracted from released virions by incubation with 1 mg/ml proteinase K at 50°C during 15 min, followed by phenol-chloroform extraction and precipitated with 3 M sodium acetate, pH 5.2, and ice-cold ethanol. This precipitate was centrifuged at 12,000 × *g* for 15 min, washed with 70% ethanol, and centrifuged again. DNA was resuspended in TE buffer (10 mM Tris-HCl and 0.5 mM EDTA, pH 8.0) and stored at 4°C for 1–2 wk until use. For restriction endonuclease (REN) analysis, viral DNA was incubated with BglII for 4 h at 37°C. Reactions were stopped by addition one-sixth volume of 6X loading buffer [0.25% (wt:vol) bromphenol blue and 40% (wt:vol) sucrose], loaded in 0.7% TAE buffer (40 mM Tris-acetate and 1 mM EDTA) agarose gels, and electrophoresed at 20 V for 12 h. Ethidium bromide-stained gels were then photographed on a UV transilluminator using Gel-Doc software (Bio-Rad, Madrid, Spain).

Results

Aerobic Counts on Microbial Contaminants. The density of microorganisms during storage differed significantly depending on the temperature and duration of storage for microbes counted at 17 h (temperature × time Pillai's trace: $F = 3.68$; df = 12, 22; $P < 0.004$) (Fig. 1a) or when reincubated for 48 h (temperature × time Pillai's trace: $F = 46.4$; df = 12, 22; $P < 0.001$) (Fig. 1b). The results of initial aerobic CFU counts (±SE) on TB agar were 1.4 (±0.17) × 10^7 CFU/ml after 17 h at 37°C, rising to 3.2 (±0.33) × 10^7 CFU/ml when reincubated for 48 h at 28°C. Additional incubation until 72 h did not result in a significant increase in CFU counts, compared with those at 48 h, and so 72 h counts are not considered further.

Aerobic CFU counts of microorganisms that contaminated the OB formulation stored at 25°C showed a steady decrease during the entire period of the study. Storage at −20°C with periodic thawing and refreezing resulted in a small decrease in CFU counts during the 18-mo period. Storage at 4°C resulted in a similar pattern of CFU counts as that observed at −20°C after 17-h incubation of plates, whereas extended incubation for 48 h at 28°C (Fig. 1b) revealed a 1 logarithm increase in CFU after 9–12 mo of storage, perhaps due to the death of mesophilic bacteria allowing slow-growing semipsychrophilic bacteria to proliferate.

Microbiological Analysis. The average total aerobic + anaerobic count of the newly formulated OB suspension was 1.9×10^8 CFU/g with $>10^7$ colonies of *Enterococcus* spp. and 7.0×10^5 of Enterobacteriaceae, and 4.5×10^3 of yeast (Table 1). After 18 mo of storage, the microbial population had changed at all temperature treatments. In the −20°C treatment, total counts had decreased by <1 logarithm, but an increase was observed in total Enterobacteriaceae and yeasts. In the 4°C treatment, total microbial populations increased by ≈1 logarithm, and total Enterobacteriaceae and yeast populations had also increased by several logarithms. Storage at 25°C resulted in marked reductions in total microbial populations, particularly the anaerobic species, total Enterobacteriaceae, and yeast. Potential human pathogens (species of *Salmonella*, *Shigella*, and *Vibrio*) were not detected, and

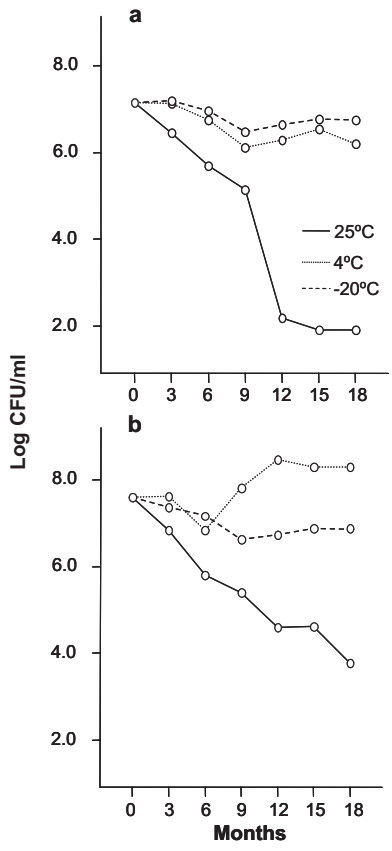

Fig. 1. Changes in aerobic CFU/ml SeMNPV occlusion body suspensions stored during 18 mo at different temperatures. Colony growth was scored after incubation of plates at 17 h at 37°C (a) and reincubated until 48 h at 28°C (b).

populations of *S. aureus* and *B. cereus* remained at the limits of detection in all treatments.

Physical Stability of OBs. The temperature of storage significantly influenced the integrity of OBs over time (time × temperature: $F = 5.34$; df = 6, 45; $P < 0.001$). The observed concentration of OBs fell by 30% after 6 mo of storage at 25°C (Table 2), and it remained statistically unchanged for the remaining 12 mo of the study. No differences were observed in the concentration of OBs between suspensions that were newly formulated and those that had been stored during 18 mo at 4 or −20°C.

Bioassays on Insecticidal Activity. The LD_{50} value of the newly formulated OB suspension in *S. exigua* second instars was estimated at 7.9 OBs per larva (Table 2). The effect of temperature on insecticidal activity after 18 mo of storage was highly significant ($F = 6.46$; df = 8, 36; $P < 0.001$). The estimated LD_{50} value of OBs stored at 25°C increased by 2.5-fold and 273-fold after 6 and 12 mo of storage, respectively. After 18 mo at 25°C, the LD_{50} exceeded the highest rate of OBs used in the bioassay, representing >16,666-fold loss of insecticidal activity. In contrast, insecticidal activity was not significantly altered after 18 mo of storage at 4°C, whereas storage at −20°C with periodic thawing to evaluate biological activity followed by refreezing resulted in a significant but small decrease in the LD_{50} value, compared with material bioassayed after 6 mo of storage (Table 2). No mortality was observed in any of the untreated controls.

Viral DNA and Restriction Endonuclease Analysis. Significant differences were observed in the genomic DNA extracted form OBs stored at different temperature conditions (Fig. 2). It was not possible to visualize DNA in agarose gels before or after endonuclease treatment in any of the three OB samples stored at 25°C, even though at least two attempts were made to extract DNA for each repetition. We conclude that this DNA was seriously degraded and could not be recovered. In contrast, DNA extracted from OBs stored at 4 or −20°C presented the characteristic profile for this endonuclease (Muñoz et al. 1997). However, the smearing observed in DNA extracted from OBs stored at 4°C suggests a degree of DNA degradation compared with DNA from OBs stored at −20°C.

Discussion

At present, the only way to produce baculovirus insecticides on an industrial scale is by using living

Table 1. Microbiological analysis of semipurified SeMNPV that was newly formulated or after 18 mo storage at different temperatures

	Newly formulated SeMNPV (CFU/g)	Microbiological analysis after 18 mo storage (CFU/g)		
		25°C	4°C	−20°C
Total plate counts	$1.9\ (\pm0.4) \times 10^8$	$2.2\ (\pm0.2) \times 10^3$	$8.4\ (\pm1.0) \times 10^8$	$4.6\ (\pm0.7) \times 10^7$
Aerobic counts (mesophilic)	$1.0\ (\pm0.5) \times 10^8$	$2.2\ (\pm0.2) \times 10^3$	$8.3\ (\pm1.0) \times 10^8$	$2.3\ (\pm0.3) \times 10^7$
Anaerobic counts (mesophilic)	$9.6\ (\pm0.4) \times 10^7$	$<10^1$	$9.6\ (\pm0.6) \times 10^7$	$2.3\ (\pm0.8) \times 10^7$
Enterococcus D	$>10^7$	$<10^1$	$>10^7$	$1.3\ (\pm0.3) \times 10^4$
Total Enterobacteriaceae	$7.0\ (\pm4.3) \times 10^5$	$<10^1$	$>10^7$	$6.9\ (\pm0.4) \times 10^6$
Yeast	$4.5\ (\pm2.4) \times 10^3$	$<10^1$	$4.7\ (\pm0.5) \times 10^7$	$2.2\ (\pm0.4) \times 10^6$
Mold	$<10^1$	$<10^1$	$<10^1$	$<10^1$
B. cereus	$<10^1$	$<10^1$	$<10^1$	$<10^1$
S. aureus	$<10^1$	$<10^1$	$<10^1$	$<10^1$
Salmonella spp.	Absent	Absent	Absent	Absent
Shigella spp.	Absent	Absent	Absent	Absent
Vibrio spp.	Absent	Absent	Absent	Absent

All values represent the mean (±SD) of three samples of SeMNPV occlusion bodies formulated with PBS, pH 6.5, sorbic acid, and glycerol.

Table 2. Occlusion body counts and logit regression of dose–mortality response of *S. exigua* second instars to newly formulated SeMNPV (0 mo) and samples stored at different temperatures for 6, 12, and 18 mo

Temp (°C) and duration of storage (mo)		Observed OB concn (±SE) (OB/ml × 10^8)[a]	Slope (±SE)	Intercept (±SE)	LD_{50} (OB/larva)[b]	95% CI		Scale parameter[c]
						Upper	Lower	
	0	6.85 (±0.28)a	1.059 (±0.13)	−10.67 (±1.33)	7.9ab	5.8	10.7	1.54
25	6	4.84 (±0.21)b	1.190 (±0.14)	−13.14 (±1.51)	20.0c	15.4	26.7	1.32
	12	5.03 (±0.11)b	0.779 (±0.09)	−12.25 (±1.81)	2172d	1456	3728	1.59
	18	4.65 (±0.35)b	-	-	>16,666[d]	-	-	-
4	6	6.55 (±0.22)a	1.038 (±0.15)	−10.46 (±1.49)	7.8ab	5.5	11.3	2.01
	12	6.45 (±0.17)a	1.113 (±0.11)	−10.89 (±1.09)	5.8ab	4.5	7.4	1.00
	18	6.59 (±0.15)a	1.142 (±0.11)	−11.12 (±1.13)	5.6a	4.2	7.1	1.00
−20	6	6.77 (±0.21)a	1.108 (±0.15)	−11.42 (±1.51)	9.9b	7.2	13.8	1.82
	12	6.49 (±0.21)a	1.140 (±0.13)	−11.40 (±1.31)	7.3ab	5.5	9.6	1.32
	18	6.79 (±0.18)a	0.963 (±0.17)	−9.042 (±1.62)	3.9a	2.4	6.1	2.63

[a] Multiple comparisons of estimated means performed following adjustment of critical α value by Bonferroni correction. Values with identical letters are statistically similar.

[b] OB concentrations used in bioassays were assumed to be unchanged from that at the start of the experiment, i.e., concentrations were not adjusted to account for loss of OBs observed in 25°C treatment.

[c] Overdispersion in dose–mortality results was taken into account by scaling the error distribution in GLIM.

[d] LD_{50} was higher than the highest dose used in bioassay: 16,666 OB/larva resulted in ≈10% virus mortality.

insects as virus factories. OB purification and formulation processes should be as simple and cheap as possible to reduce costs, and these will obviously influence the physical and biological properties of formulated OBs in storage, distribution, and application. Apart from the difficulties of handling very large numbers of insect larvae, the presence of contaminant microorganisms is probably the most important problem of baculovirus production. Regulatory authorities in Europe do not always have a clear or coordinated definition of the quantities of microbial contaminants that are permissible in a microbial insecticide (Jenkins and Grzywacz 2000), whereas the United States IR-4 program has more clearly defined regulatory criteria for biopesticides that favor the registration of these products (EPA 2007).

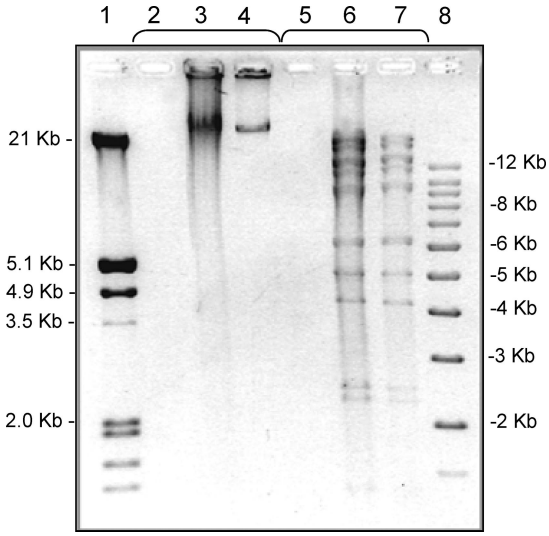

Fig. 2. Agarose electrophoresis of DNA isolated from OBs (pooled samples from three repetitions) after 18 mo of storage at 25, 4, and −20°C (lanes 2, 3, and 4, respectively), and BglII restriction analysis of the same DNA after endonuclease treatment (lanes 5, 6, and 7). Molecular size markers were lambda DNA/EcoRI+HindIII marker (lane 1) and 1-kb DNA ladder (lane 8).

The newly formulated OB suspension contained an average of 1.9 (±0.5) × 10^8 CFU/g, composed of ≈50% aerobes and 50% anaerobes. These values are similar to the results of Smits and Vlak (1988) who reported between 3 × 10^7 and 1 × 10^8 CFU aerobes per larva in insects dying from SeMNPV at 6–10 d postinfection. Maximum levels of aerobic bacteria established for baculovirus insecticides in the United States were initially rigorous with 10^7 CFU/g in the *Helicoverpa zea* (Boddie) nucleopolyhedrovirus (NPV) marketed under the name Elcar (Couch and Ignoffo 1981). Products developed later had more realistic limits, such as 10^9 CFU/g for the gypsy moth, *Lymantria dispar* L., NPV and Douglas fir tussock moth, *Orgyia pseudotsugata* (McDunnough), NPV (Shapiro 1986). Microbial populations can vary widely in different batches of NPV: an average of 6 × 10^8 CFU/g was observed in batches of *L. dispar* MNPV (Podgwaite et al. 1983), whereas values of ≈4 × 10^6 CFU/g were reported by Shapiro et al. (1981) for the same virus produced in the United States. Similarly, aerobic microbial counts varied between 4.4 × 10^6 and 4.5 × 10^8 CFU/ml in samples taken during a 6-mo period of production of *Spodoptera littoralis* (Boisduval) NPV in an Egyptian laboratory (Grzywacz et al. 1997). Jenkins and Grzywacz (2000) have pointed out that bacteria proliferate rapidly after death of an infected insect and that a target of 1 × 10^8 CFU/ml (aerobic conditions) for liquid formulations or 5 × 10^8 CFU/g for dry powders is likely to be both safe and attainable. The majority of these studies have focused on microorganisms associated with recently harvested OB preparations in contrast to the current

study that examined changes in microbial loads during storage of a formulated product containing a microbiostatic adjuvant, namely, sorbic acid.

The majority of microbial contaminants were not identified to species but the dominant *Enterococcus* group probably consisted mainly of harmless species belonging to the normal gut flora of the larvae, as observed in other studies on recently harvested samples of OBs (Krieg et al. 1979, Smits and Vlak 1988). Certain strains of *Enterococcus* species, such as *E. faecalis* and *E. faecium*, can be opportunistic intestinal pathogens, but these species are common contaminants of OB preparations and do not normally represent a hazard to human health (Grzywacz et al. 1997). Similarly, members of the Enterobacteriaceae include a number of potential human pathogenic species from genera such as *Klebsiella*, *Citrobacter*, and *Enterobacter* that are also commonly associated with laboratory-reared insects.

Although not considered but most studies on baculovirus contaminants, the results of the anaerobic analyses reported here indicated that the presence of anaerobes or microaerobes are not likely to give cause for concern in baculovirus-based biopesticides. No vertebrate pathogens such as, *Vibrio* spp., *Shigella* spp., and *Salmonella* spp., were detected in any of our SeMNPV samples. Moreover, no other human pathogens were detected in other batches analyzed from a total production of 300 liters during the course of 10 mo (data not shown). Vertebrate pathogens have not been found in other baculovirus production systems (Shapiro 1982, Smits and Vlak 1988), with the exception of the sporadic occurrence of *B. cereus* (Podgwaite et al. 1983, Grzywacz et al. 1997). A healthy insect colony and hygiene of workers are necessary to minimize contaminants like *B. cereus*, for which some toxin-producing strains can cause food poisoning if present at high concentrations (10^5-10^6 CFU/g) or *S. aureus*, which commonly inhabits the human skin surface and nasopharynx, but can cause abscesses of the skin and eyes (Kramer et al. 1982, Garcia-Lara et al. 2005). High contaminant levels in virus produced in insects may be indicative of a high bacterial load present in the inoculum used for the infection process. This can be overcome by using highly purified inoculum, strict hygiene procedures and by harvesting dying and recently dead insects at frequent intervals to avoid the proliferation of bacteria post mortem.

The activity of SeMNPV stored under refrigerated or frozen conditions was comparable that of newly formulated material. In contrast, the insecticidal activity was compromised after 6 mo of storage at 25°C and eliminated after 18 mo storage at this temperature. The processes that lead to loss of baculovirus infectivity during storage are poorly understood. Deterioration may be due hydrolysis and autoxidation as result of exposure to oxygen derived from lipids that are present in insect cadavers and that have not been removed by simple filtration (Burges and Jones 1998). This may lead to proteolysis or production of free radicals. Ignoffo and Garcia (1994) showed that free radicals and superoxides generated during autoxidation are capable of disrupting the structure of nucleic acids and are potential causes of loss of pathogenicity in stored virus. Our results suggest that the DNA of occluded virions is highly degraded during storage at 25°C. Although OBs were observed to be present, probably only small fragments of DNA remained inside the occluded virions that could not be visualized by electrophoresis and ethidium staining. In contrast, DNA purified from OBs that had been stored refrigerated or frozen did not suffer degradation.

Studies on long-term storage indicate that nucleopolyhedrovirus preparations are affected by the method of extraction, purification, formulation and storage conditions. Generally, stability in storage is improved by reducing the quantity of unsaturated fats and limiting the availability of oxygen, and by storing at low temperatures and away from light. For example, spray dried formulations of the *Anagrapha falcifera* NPV lost insecticidal activity when storage up to 1 yr at room temperatures (Tamez-Guerra et al. 2002). Nevertheless, in the same experiment, unformulated virus stock did not lose activity at ambient or refrigerated temperatures. *O. pseudotsugata* MNPV loss 46% of infectivity after 2 yr of storage at 4°C and 44% RH in plastic bags (Kaupp and Ebling 1993). In contrast, no significant losses were observed in the same virus after 14 yr of storage in vacuum packages at −10°C, and no adverse effects on the quality of OpMNPV DNA were observed after prolonged cold storage (Reed et al. 2003, Otvos et al. 2006). The disappearance of OBs stored at 25°C is likely due to proteolysis during the period immediately after formulation, before proteases had degraded or been inactivated. Clearly, such enzymatic activity was minimized or eliminated in low temperature storage.

Our results agree with those of Cherry et al. (1996) who concluded that bacterial contamination does not have a direct impact on the shelf life of baculovirus based insecticides, although it may be implicated in accelerating the chemical processes that inactivate OBs. Baculovirus OBs can remain stable for several years at room temperature in highly purified suspensions, but exhaustive purified methods are an expensive step in the production process and are best avoided (Shapiro 1982). In the case of the SeMNPV product that is being produced for field testing in Spain, storage, and distribution in refrigerated conditions will likely favor a product shelf life of over 18 mo. However, special conditions for the storage, transport and use of a biopesticide should not be regarded as an alternative to appropriate formulation (Burges and Jones 1998). Studies into improving the cleanliness of the virus production process and the incorporation of additional bacteriostatic or antioxidant additives may be necessary for the development of an SeMNPV-based insecticide that can remain stable over several years of storage.

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