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Full Length Research Paper

Characterization of baculovirus isolates obtained from soil by restriction fragment patterns

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Four baculovirus isolates recovered from soil in corn plots infested with fall armyworm (FAW) larvae were characterized using three different restriction enzymes. Each isolate was amplified *in larvae* in aseptic conditions, to increase the number of occlusion bodies (OBs). Baculovirus isolates were purified on continuous sucrose gradients by ultracentrifugation. The purified DNA of each isolate was digested with restriction enzymes *Eco*RI, *Hind*III and *Bam*HI. Each isolate showed a significant difference in the fragments obtained by electrophoresis. Isolates CAD and NAV_1 showed similar band patterns, the AN_1 and AN_2 isolates had different patterns between them and the others. Virulent isolates of baculovirus can be obtained from soil, which can be an important reservoir of these entomopathogenous viruses.

Key words: Baculoviridae, nucleopolyhedrovirus, granulovirus, fall armyworm, restriction enzymes.

INTRODUCTION

Actually, a diversity of baculovirus has been demonstrated by the characterization of different geographic isolates, especially with the genotypic variants present within the same isolate. Knowledge of the natural diversity, inter- and intra-specific, could contribute to a better classification of baculovirus, as well as being of special importance for the design of bio-insecticides, whose genetic material could be included in the improvement of strains with better potential for application in specific agroecosystems (Muñoz et al., 2001). Baculoviruses are classified according to the host from which was isolated, although they are highly specific, there are baculovirus infecting several host species, making reference to them by different names (Harrison and Bonning, 1999). Also, isolates from the same host species may be different baculoviruses, since in a single sample can be found even two types of nucleopolyhedrovirus (multiple MNPV and simple SNPV) (Li et al., 2002). Essentially, all isolates used against fall armyworm (FAW) larvae have been isolated from infected insects.

There are no prior reports on obtaining and using baculovirus isolated from soil, and few have been characterized in detail. Baculoviruses are often identified by their characteristic morphology and symptoms in their hosts. It is possible to routinely characterize baculovirus by obtaining their restriction fragment patterns and so differentiate their genomic DNA between species or isolates. In the present trial we reported the isolation, purification and molecular characterization using three different restriction enzymes, in four different native baculovirus isolates obtained from soil in a corn field infested with FAW larvae in Coahuila and Nuevo León

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states, in Mexico.

MATERIALS AND METHODS

Insect colony

FAW larvae were obtained from a colony established under controlled conditions (25 ± 2°C, 12:12 L:D photoperiod and 50 to 60% RH). Larvae were reared on an artificial diet (Southland Products Incorporated).

Baculovirus isolation and amplification

Baculovirus isolates were recovered from soil of corn plots in the states of Coahuila and Nuevo Leon, Mexico. Soil samples were taken by scraping away the upper five centimeters of soil, and then collecting soil at depths of 5 to 10 cm. Each sample consisted of 600 to 800 g soil. Samples were processed according to the methodology described by Richards and Christian (1999). Thus each 25 g sample of sieved soil was incorporated in 100 ml of artificial diet where first and second FAW instars were placed on the amended diet. Larvae that died of polyhedrosis and/or granulose disease were considered to be infected by a baculovirus isolate. These baculovirus isolates were amplified *in vivo* in FAW third instar and purified by filtration and centrifugation as described by Muñoz et al. (2001). The concentration of viral occlusion bodies (OBs) were quantified with a hemacytometer and stored in aliquots of 500 µL of sterile distilled water at 0°C until required.

Insecticidal activity of native isolates of baculovirus

The median lethal concentration (LC_{50}) of each viral isolate recovered was determined by the diet surface contamination technique. The diet surface in each container was inoculated with 1 of 7 baculovirus concentrations ranging from 2.0×10^{1} to 4.0×10^{6} OBs/mm^2; and 20 FAW larvae per concentration were infected in each of 3 replicates. Larvae used as control in the bioassay were placed in cups with artificial diet treated only with sterile distilled water. Mortality was measured daily for 25 d. Mortality was corrected by Abbott's formula (Abbott, 1925), and the means of treatments were separated using the Tukey's test ($P<0.05$). The LC_{50} values were calculated by the Probit method using the Statistical Program SAS (SAS, 2002).

Purification of occlusion bodies and virions

Purification of OBs of baculovirus was performed using continuous gradients of sucrose from 40 to 66% w/w, using a gradient forming tube double (Bethesta Research Laboratories). Highest sucrose concentration was added in the inner tube, and the lowest concentration in the outer tube. The gradient was collected in 50 ml pollyalomer tubes and centrifuged at 24,000 rpm in an ultracentrifuge (Beckman L8-70M), at 4°C for 1 h with a SW 28 rotor. From the bands formed in the pollyalomer tubes were collected the OBs (polyhedra and/or granules) with Pasteur pipettes and transferred to new polypropylene tubes with 20 ml of sterile distilled water and were washed by centrifugation at 10,000 rpm and finally resuspended in 500 µL aliquots of sterile distilled water and stored at 4°C until used.

For the release of virions, at OBs previously purified were added 300 µL of alkali (0.1 M Na_2CO_3, 0.1 M NaCl, pH 10.8) and 100 µL of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6), incubated at 25°C with agitation at 140 rpm for 90 min. Released virions were purified on continuous sucrose gradients from 20 to 60% by ultracentrifugation at 28,000 rpm for 1.5 h. Later blue bands containing the virions were collected with a Pasteur pipette and washing by centrifugation was performed at 28,000 rpm for 40 min. The virions were stored in Eppendorf tubes at 4°C until used.

DNA extraction and digestion with restriction enzymes

To extract DNA, the purified virions were treated with 400 µL of buffer for proteinase K (0.01 M Tris, 0.005 M EDTA, 0.5% SDS) and 100 µL of a proteinase K solution (200 µg/ml) and incubated for 2 h in a water bath at 37°C. Subsequently, DNA extraction was carried out by adding 500 µL of the solution phenol: chloroform: isoamyl alcohol (25: 24: 1), and centrifuged at 13,000 rpm for 5 min, the aqueous phase was collected and transferred to another tube. It added 500 µL of Isopropanol, incubated for 10 min at 4°C and centrifuged at 13,000 rpm for 10 min; the pellet collected from this process was resuspended with 30 µL of sterile distilled water and stored at -20°C, until used. The DNA of each isolate was digested separately with three restriction enzymes, *Eco*RI, *Hind*III and *Bam*HI incubating the reaction for 2 h at 37°C. The DNA was run on agarose gel 0.6% at 30 V for 18 to 24 h in a horizontal electrophoresis chamber Maxicell with TBE (Tris Base 0.089 M, boric acid 0.089 M, EDTA 0.0025 M, pH 8.0) as reaction buffer. The molecular weight marker 1 kb Ladder (Gibco BRL) was used. DNA fragments in the gel were stained with ethidium bromide (0.5 µg/ml) and examined with an UV transilluminator.

RESULTS AND DISCUSSION

Samples of baculovirus-infected larvae were macerated, an aliquot of the resulting suspension was observed under a phase-contrast microscope (Carl Zeiss), where typical OBs of variable size, (polyhedra and granules) characteristic of the Baculoviridae family were observed. It was found that three of the four soil isolates are a mixture of the two genera of Baculoviridae: Granulovirus and Nucleopolyhedrovirus (Figure 1). In this regard Cory et al. (2005), mentioned that NPV isolates from individual larvae rarely contain a single genotype or variant, as shown in the images observed under the microscope, so we can say that soils from corn plots infested with FAW larvae are the major natural reservoir of OBs from two genera of baculovirus (NPV and GV) (Richards and Christian, 1999). As shown in Figure 1, baculovirus isolates obtained from soil, showed the presence of at least two types of viral OBs (polyhedra and granules).

The four geographic isolates of baculovirus tested against third instar FAW larvae have shown to be pathogenic, but there were variations in virulence levels, as reflected in the LC_{50} (Table 1), with the AN_2 isolate (from the experimental field of Saltillo, Coahuila), which showed the highest biological activity, since the estimated LC_{50} to third instar FAW larvae was 5.7×10^{2} OBs/mm^2, 100 times less than that shown by the AN_1 isolate. The AN_1 isolate was the second more virulent, with 6.5×10^{4} OBs/mm^2. This bio-insecticide activity level is similar to isolates described by Martínez et al. (2003), as reported a LC_{50} of 3.4×10^{4} OBs/ml in the same instar larvae. In NAV_1 and CAD isolates (Table 1), the LC_{50} estimated were 1.2×10^{5} and 1.9×10^{5} OBs/mm^2,

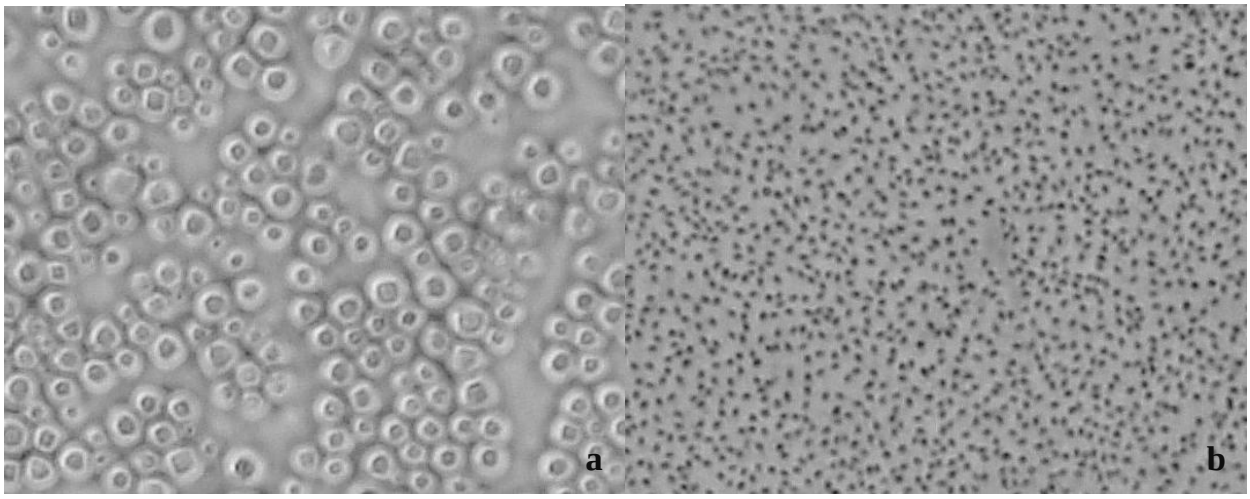

Figure 1. Occlusion bodies (OBs) of baculovirus obtained from the purification of *S. frugiperda* larvae; (a) polyhedra, and (b) granules. Microphotographies at 100×.

Table 1. Median lethal concentration $LC_{50,}$ of native baculovirus isolates on third instar larvae of *S. frugiperda*.

Isolate[a]	N[b]	% Mortality[c]	Lower limit	LC_{50} (95%)[d]	Upper limit	Slope (±SE)	Intercept (±SE)	χ^2	R^2
NAV_1	420	52.63	6.7×10^4	1.9×10^5c	9.5×10^5	0.52 ± (0.07)	-2.73 ± (0.34)	0.98	0.86
CAD	420	80.00	9.7×10^4	1.2×10^5c	1.4×10^5	1.25 ± (0.13)	-6.34 ± (0.67)	0.83	0.98
AN_1	420	72.22	5.4×10^4	6.5×10^4b	7.7×10^4	1.53 ± (0.18)	-7.35 ± (0.90)	0.87	0.96
AN_2	420	100.00	4.3×10^2	5.7×10^2a	7.2×10^2	2.86 ± (0.34)	-7.92 ± (0.97)	0.99	0.95

[a]Baculovirus isolates, NAV1: isolated from corn plots at Navidad, Nuevo León; CAD isolated from Cadereyta, Nuevo León; AN1 and AN_2: isolated from Saltillo, Coahuila, Mexico. [b]Number of insects treated. [c]Percent mortality with the highest concentration (4.0×10^6 OBs/mm^2). [d]LC_{50} values were expressed as OBs/mm^2 on the diet surface. Twenty larvae per NPV concentration, 7 concentrations per isolate and 3 replicates; 20 untreated larvae (control) per replicate were used. SE = Standard error; χ^2 = Goodness of fit test. R^2 = Coefficient of determination.

respectively, lower activity than which was published by Vasquez et al. (2002), who found a LC_{50} of 4.9×10^4 OBs/ml.

It is noteworthy that with the exception of AN_2, the isolates contained a mixture of granules and polyhedra (GVs and NPVs). A recent study with NPV (PfNPV) from *Panolis flammea* showed that the inoculum containing more than one variant was more pathogenic than those having a single (Hodgson et al., 2004). However, in this trial, the highest levels of virulence were found in AN_2, isolate that had not mixture (Table 1), as evidenced by morphological studies. According to molecular characterization by obtaining patterns of restriction fragments with different enzymes, was observed that the isolates differ in their DNA restriction fragments. Three of the four isolates (AN_1, AN_2 and CAD-NAV_1) showed different profiles which may mean different OBs strains, depending on the soil origin (Figure 2). NAV_1-CAD isolates showed very similar profiles with the restriction enzymes *Eco*RI and *Hind*III (Figure 2) and coincidentally, also had statistically similar virulence (Table 1). However, these patterns were different from AN_1 and AN_2 isolates digested with the same enzymes. Likewise, AN_1 and AN_2 baculoviruses from Saltillo, Coahuila, had different patterns between the three restriction enzymes used, and their levels of virulence were statistically different, with AN_2 being the most pathogenic isolate (Table 1).

DNA bands generated by these two isolates using the enzymes *Bam*HI, *Eco*RI and *Hind*III were different, so possibly both isolates belong to two different strains. Differences in DNA fragments patterns obtained using *Eco*RI, *Hind*III and *Bam*HI showed that three of the four isolates are different (AN_2, AN_1, NAY_1-CAD), the same way that they behave in their mortality to *Spodoptera frugiperda.* Three of the four baculovirus isolates (AN_1, NAV_1 and CAD) are mixtures of NPVs and GV_S, while AN_2 isolate containing only polyhedra of NPV genera. Isolates showed different levels of biological activity in the same host species and showed different banding patterns (groups of genes) so feasibly are different genotypes of baculovirus. In nature, infections with baculoviruses mixture are not frequently observed. However, they are found in Lepidoptera, with both synergistic and antagonistic activity (Krieg, 1971). In

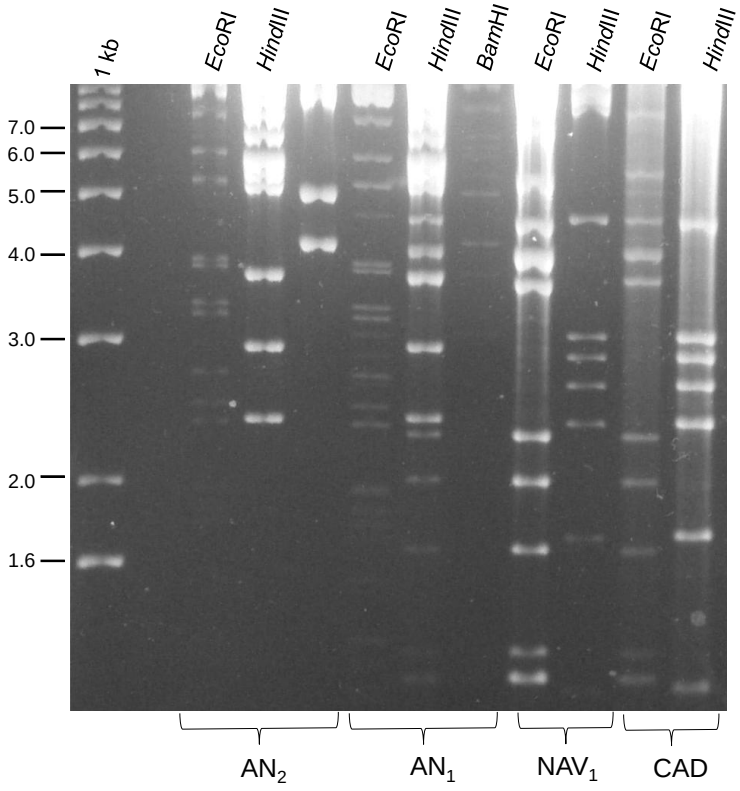

Figure 2. DNA fragment analysis of four isolates of baculovirus digested with three restriction enzymes (*Eco*RI, *Hind*III and *Bam*HI). The designation of isolates found in Table 1.1 kb ladder.

different isolates from *S. frugiperda*, Smits and Vlak (1988) found that small genotypic differences between them did not affect its biological activity. In this study isolates with similar restriction patterns showed differences in their levels of virulence. So probably, the different bands may contain genes involved in the virulence.

REFERENCES

Abbott WS (1925). A method of computing the effectiveness of an insecticide. J. Econ. Entomol., 18(2):265-267.

Cory JS, Green BM, Paul RK, Hunter-Fujita F (2005). Genotypic and phenotypic diversity of a baculovirus population within an individual insect host. J. Invert. Pathol., 89: 101–111.

Harrison RF, Bonning BC (1999). The nucleopolyhedroviruses of *Rachoplusia ou* and *Anagrapha falcifera* are isolates of the same virus. J. Gen. Virol., 80: 2793–2798.

Hodgson DJ, Hitchman RB, Vanbergen AJ, Hails RS, Possee RD, Cory JS (2004). Host ecology determines the relative fitness of virus genotypes in mixed genotype nucleopolyhedrovirus infections. J. Evol. Biol., 17: 1018–1025.

Krieg A (1971). Interactions between pathogens. p 459-489 In: Burges HD, Hussey NW (eds) Microbial control of insects and mites. Academic Press, New York, pp. 861.

Li Q, Donly C, Li L, Willis LG, Theilmann DA, Erlandson M (2002). Sequence organization of the *Mamestra configurata* nucleopolyhedrovirus genome. Virology, 294: 106–121.

Martínez AM, Simón O, Williams T, Caballero P (2003). Effect of optical brighteners on the insecticidal activity of a nucleopolyhedrovirus in three instars of *Spodoptera frugiperda*. Entomol. Exp. Appl., 109: 139-146.

Muñoz D, Mabel MA, Murillo R, Ruíz EI, Vilaplana L (2001). Técnicas básicas para la caracterización de baculovirus, p. 478-518. In Caballero P, Williams T, López M (eds) Los baculovirus y sus aplicaciones como bioinsecticidas en el control biológico de plagas. Phytoma, España, pp. 518.

Richards AR, Christian PD (1999). A rapid bioassay screen for quantifying nucleopolyhedroviruses (Baculoviridae) in the environment. J. Virol. Meth., 82: 63–75.

SAS INSTITUTE (2002). “SAS User´s Guide. Version 9.0.” SAS Institute, Cary, NC USA.

Smits PH, Vlak JM (1988). Selection of nuclear polyhedrosis viruses as biological control agents of *Spodoptera exigua* (Lepidoptera: Noctuidae). Entomophaga, 33(3): 299-308.

Vásquez J, Zeddam J, Tresierra AA (2002). Control biológico del “cogollero del maíz” *Spodoptera frugiperda*, (Lepidoptera: Noctuidae) con el baculovirus SfVPN, en Iquitos, Perú. Folia Amazón., 13: 25-29.