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Population genetic structure determines speed of kill and occlusion body production in *Spodoptera frugiperda* multiple nucleopolyhedrovirus

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Abstract

A Nicaraguan isolate of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfNIC) survives as a complex mixture of genotypes (named A to I). The speed of kill, time-mortality distribution, and occlusion body (OB) production of single genotypes (A, B and F) and co-occluded mixtures of genotypes, in a 75% + 25% ratio, were compared to determine the contribution of each genotype to the transmissibility of the viral population. Pure genotypes differed markedly in their speed of kill in second instar *S. frugiperda*. The speed of kill of SfNIC was attenuated compared to that of the dominant genotype B, indicating that interactions involving two or more genotypes likely determine host killing traits in the virus population. Genotypes A, F and defective genotype C, had no significant effects on the distribution of insect deaths over time when present as minority components in mixtures comprising 75% of genotype B. Similarly, the mortality pattern over time of insects infected by genotype F, the fastest-killing genotype tested, was not affected by the presence of genotypes A or C. Semi-quantitative PCR studies indicated that the genetic composition did not differ significantly between SfNIC-infected insects that died soon (67 h) or late (139 h) after inoculation, suggesting that stability in genotypic composition is important for virus survival. Median OB production per insect was correlated with mean time to death so that attenuated speed of kill of SfNIC resulted in high OB yields. We conclude that (i) minority genotypes play a functional role in determining the timing of mortality of infected hosts and (ii) the genotypic structure of the virus population is stably maintained to maximize the likelihood of survival.
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Keywords: Genotypes; Baculovirus; Occlusion body production; SfMNPV; Speed of kill; Transmissibility

1. Introduction

Studies on genotypic heterogeneity within baculovirus populations by *in vitro* (Lee and Miller, 1978; Maruniak et al., 1984) or *in vivo* (Smith and Crook, 1988; Muñoz et al., 1998) techniques have revealed that natural populations often comprise a number of different genotypes that may differ in the presence of certain genes (Durantel et al., 1998; Simón et al., 2004; Cory et al., 2005). Certain genotypic variants may also be more prevalent than others in wild-type populations (Muñoz et al., 1998; Hitchman et al., 2007). The fact that minority genotypes are not eliminated from the population suggests that this heterogeneity is important for virus survival (Possee and Rohrmann, 1997).

An insect that consumes a nucleopolyhedrovirus (NPV, genus *Nucleopolyhedrovirus*, family Baculoviridae) occlusion body (OB) ingests multiple virions, and for the multi-

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ply enveloped NPVs, a single virion may contain multiple genomes (Theilmann et al., 2005). Multiple encapsidation is a way of overcoming the host midgut epithelial cell sloughing response to viral infection, because some of the nucleocapsids from multicapsid virions are immediately repackaged and exported from the epithelial cell as budded virus to initiate systemic infection (Washburn et al., 1999). This infection strategy significantly increases the probability of successfully establishing an infection following the ingestion of OBs (Washburn et al., 2003). This has important consequences for the genetic structure of NPV populations as genotypically different virions can be occluded in a single OB, so that an individual insect is likely to be infected by multiple genotypes. Following the primary infection, it has been demonstrated that, on average, each cell in an insect is infected by multiple virions (Bull et al., 2001), so that the genotypic diversity in each cell of the secondary infection likely reflects the diversity present in the primary infected cell(s).

A Nicaraguan isolate of a multiple NPV (SfNIC) of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) has been selected for formulation and field evaluation for control of *S. frugiperda* by Mesoamerican maize growers (Castillejos et al., 2002). The SfNIC isolate survives as a complex mixture of at least nine genotypes SfNIC-A to SfNIC-I (hereafter referred to as genotypes A to I) (Simón et al., 2004). Genotype B was the predominant genotype in the SfNIC population and its genome size was calculated to be around 129 kbp (Simón et al., 2005a). All the other genotypes displayed one or more deletions compared to genotype B. Genotypes F, G, and I had a deletion of around 7 kb, genotypes A, E, and H had a deletion of around 10 kb, and genotypes C and D had a deletion of around 15 kb (Simón et al., 2004, 2005c).

Deletion genotypes C, D and G are not able to infect *S. frugiperda* larvae by ingestion (Simón et al., 2004). The absence of oral infectivity of genotypes C and D is related to the deletion of the *pif* and/or *pif*-2 genes that are essential for cell binding and penetration during the process of infection of insect midgut cells (Kikhno et al., 2002; Pijlman et al., 2003; Simón et al., 2005b). None of the *per os* infectious genotypes is as pathogenic (*sensu* Thomas and Elkington, 2004) as the wild-type population, suggesting that interactions among genotypes result in altered pathogenicity, as measured by 50% lethal concentration assays (LC_{50}). This hypothesis was briefly confirmed in a previous study, where it was shown that the mixtures B+A, B+C, B+D, B+F showed increased pathogenicity compared to single genotypes alone (López-Ferber et al., 2003; Simón et al., 2005c). However, only the combination of B+C and B+D restored the pathogenicity of the mixture to that of the natural population when mixed in approximately the same proportion (75% + 25%) as observed in the wild population.

Phenotypic characteristics, such as speed of kill, differ between genotypes so that genotypic composition will affect the efficacy of the pathogen as a biological insecticide. Our aim in this study was to compare virus-induced mortality patterns over time and OB production of single genotypes and mixtures of genotypes in order to determine the contribution of each genotype to these phenotypic traits and their likely influence on the survival of the virus population.

2. Materials and methods

2.1. Insects and viruses

Larvae of *S. frugiperda* were obtained from a laboratory colony continuously maintained in a growth chamber at 25 ± 1 °C, 75 ± 5% RH and a photoperiod of 16 h light and 8 h dark on semisynthetic diet (Greene et al., 1976). A Nicaraguan isolate of *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV-NIC) was originally isolated from diseased *S. frugiperda* larvae infesting maize plants in Nicaragua (Escribano et al., 1999). Viral occlusion bodies (OBs) of this isolate (SfNIC) were produced by inoculating fourth instars by the droplet feeding method (Hughes and Wood, 1981). OBs were extracted from dead diseased larvae by homogenization in water and purified by filtration and differential centrifugation. OBs were stored at 4 °C in distilled water until used. OB concentration was determined using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase-contrast microscopy.

The SfNIC genotypic variants used in this study had been cloned by plaque purification as previously described. Nine different genotypes were purified (Simón et al., 2004). Genotypes SfNIC-C, -D and -G, that are not infectious *per os*, were produced by injection of occlusion derived virions (ODVs) in larvae, whereas the infective genotypes were amplified by oral inoculation of larvae.

For extraction of viral DNA, virions were released from a suspension comprising ~10^9 OBs by addition of 100 μl of 0.5 M Na_2CO_3 and 50 μl of 10% w/v SDS in a final volume of 500 μl and were incubated at 60 °C for 10 min. Undissolved OBs were pelleted by low-speed centrifugation (2700*g*, 5 min). The supernatant containing the ODVs, was treated with 25 μl of proteinase K (20 mg/ml) for 15 min at 50 °C. Viral genomic DNA was extracted twice with TE buffer-saturated phenol and once with chloroform, and precipitated by addition of sodium acetate, pH 5.2, to a final concentration of 0.3 M, and 500 μl of ice-cold ethanol (final concentration 70%). This was centrifuged at 3800*g* for 10 min, washed with 70% ethanol and dissolved in 0.1× TE buffer. DNA concentration was estimated by absorbance at 260 nm.

2.2. Production of genotype mixtures

Genotype mixtures were produced by injecting larvae with mixtures of the following combinations of genotypes: B+A, B+C, B+F, A+C, F+C, A+F and F+A as previously described (López-Ferber et al., 2003; Simón et al.,

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