Genetic identification and biological characterization of Baculovirus isolated from *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Brazil

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Abstract

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) larvae are polyphagous, aggressive, and have been found in many of Brazil's agricultural areas. Biological control with baculoviruses is part of an integrated pest management (IPM) strategy to manage this insect. Three isolates of nucleopolyhedrovirus (NPV) were collected from Brazilian populations of *H. armigera*, and compared genetically and biologically to Gemstar® (an imported nucleopolyhedrovirus), and to *Nucleopolyedrovirus spodoptera* (SPNPV) that was passed serially through *H. armigera*. Genetic sequencing of lef-8 and lef-9 genes revealed that the Brazilian isolates were closely related to nucleopolyhedrovirus species from Australia, South Africa, China, and India. The isolates caused high mortality rates in third instar *H. armigera* larvae. The mean lethal dose (LD_{50}) and lethal time (LT_{50}) differed between isolates, but was highest for HearNPV-BR2. This is the first report of HearNPV in Brazil, and the insecticidal properties of the BR2 isolate support its potential use in the production of biopesticides to manage *H. armigera* in Brazil.

Key Words: biological control; nucleopolyhedrovirus; vertical transmission; virus adaption

Resumo

Lagartas de *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) são polífagas, agressivas e foram identificadas no Brasil atacando extensas áreas de produção agrícola. O controle biológico de baculovírus faz parte do manejo integrado de pragas (MIP). Três isolados de nucleopoliedrovírus (NPV) foram coletados em populações brasileiras de *H. armigera* e comparados geneticamente e biologicamente, utilizando Gemstar®, com base em um nucleopoliedrovírus importado e multiplicação seriada de *Nucleopolyedrovirus spodoptera* (SPNPV) em *H. armigera*. Foram realizados testes genéticos e biológicos entre as estirpes autóctones, comparando-as com o produto comercial Gemstar®. A análise comparativa do sequenciamento genético realizada para os genes lef-8 e lef-9 revelaram que os isolados locais estão, estreitamente, relacionados com espécies de baculovírus da Austrália, Índia, África do Sul e China. Todos os isolados testados possibilitaram o controle de lagartas de terceiro instar de *H. armigera*. Analises biológicas da dose letal média (DL50) e do tempo letal médio (TL50) variaram entre os isolados testados. O isolado HearNPV-BR2 apresentou os melhores resultados de DL50 e TL50. Ademais, este é o primeiro registro da ocorrência da espécie HearNPV no Brasil, e suas propriedades inseticidas assinalam que a mesma pode ser útil para a fabricação de bioinseticidas para o controle de *H. armigera* no país.

Palavras Clave: controle biológico; nucleopoliedrovírus; transmissão vertical; adaptação de bacuvírus

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a polyphagous insect, and one of the most important agricultural pests worldwide (Cunningham & Zalucki 2014). Its larvae preferentially feed on the reproductive structures of agricultural plants (Fitt 1989). Economic losses by *H. armigera* are estimated to be more than $2 billion annually, in addition to socio-economic and environmental costs (Tay et al. 2013). *Helicoverpa armigera* now has spread through most of Brazil, and is present in Paraguay and Argentina, and recently was detected (though not established) in Florida, USA (Czepak et al. 2013; Specht et al. 2013; Leite et al. 2014; Murúa et al. 2014; Hayden & Brambila 2015).

Resistant cultivars expressing *Bacillus thuringinsis* (Bt) proteins, and chemical insecticides, are the basis of integrated management of *H. armigera*. However, resistant populations have been identified in Australia, China, and India (Tabashnik et al. 2009; Liu et al. 2010; Yang et al. 2013). Baculovirus-based biopesticides stand out as an important potential control method that could be used in combination with other pesticides (Moscardi 1999; Raymond et al. 2006). Baculoviruses infect insects and are considered sophisticated pathogens used for biological control (Rollie et al. 2013). Baculovirus-based insecticides have high specificity, virulence, the ability to persist in the environment, and compatibility with natural enemies (parasitoids,

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pathogens, and predators) and chemical insecticides. Alphabaculovirus-based biopesticides are successful in controlling Lepidoptera pests such as *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) and *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) (Moscardi 1999; Kutinkova et al. 2012).

It is important to identify virus strains with the greatest potential for biological control prior to manufacturing a bioinsecticide (Figueiredo et al. 2000; Chen et al. 2002; Arrizubieta et al. 2013). This is essential because virus toxicity varies with host type and collection site (Milks 1997; Barrera et al. 2011); in addition, non-native isolates can reduce the activity of local strains (Muñoz & Caballero 2000). Furthermore, Haase et al. (2015) reported that the search for local isolates is necessary to increase the number of isolates available and to find the most effective isolate for each pest in each region. Baculovirus products based on foreign isolates have been registered for commercial use against *H. armigera* in Brazil. The objective of this study was to identify and study the toxicity of local baculovirus strains (isolated in Brazil) to *H. armigera* larvae.

Materials and Methods

Larvae of *H. armigera* were obtained from the Biological Control Laboratory of Applied Biology Center "Embrapa Milho e Sorgo" in Sete Lagoas, Minas Gerais State, Brazil. This insect was reared in a room at 25 ± 2 °C, with 70 ± 10 % RH, and a 12:12h (L:D) photoperiod, and fed an artificial white bean-based diet (Greene et al. 1976).

Five virus isolates were obtained; 3 were from *H. armigera* larvae, initially without signs of viral infection, collected from corn plantations in Sete Lagoas, Minas Gerais State, Brazil. These larvae were taken to the laboratory where they showed typical baculovirus infection signs. Three larvae (1 collected in Feb 2013 and 2 in Apr 2014) showed behavioral changes, including feeding reduction, and morphological signs such as decreased growth and discoloration of the integument. These insects died within a few d of the integument rupturing. The other 2 strains were obtained from the commercial product Gemstar®LC, effective in the control of *H. armigera* (HzSNPV) or *Baculovirus spodoptera* SpNPV-BR4. One was considered the reference isolate, whereas the other was obtained by serial passage of a *Baculovirus spodoptera* SpNPV-BR4 through *H. armigera* larvae until it caused 100% mortality (Pavan et al. 1989). This isolate, *Spodoptera frugiperda* SfNPV, which was isolated from *Spodoptera frugiperda* Smith & Abbot (Lepidoptera: Noctuidae) larvae, was obtained from the "Embrapa Milho e Sorgo" baculovirus bank, and sprayed on corn leaves containing *H. armigera* larvae. This baculovirus was multiplied 3 times in *H. armigera* populations, a procedure known as serial passage. This isolate caused 100% larval mortality in the third passage, and was therefore used in the bioassays.

Five virus isolates (Table 1) were purified by sucrose gradient centrifugation (Caballero et al. 1992), and multiplied in 112 third instar *H. armigera* larvae fed 1.8 × 1.8 cm corn leaf discs impregnated with baculovirus suspensions at a dosage of 1×10^9 polyhedra per mL per isolate. The dead insects were macerated in 100 mL distilled water and strained using a thin layer of cotton (Gomez et al. 1999). The polyhedra obtained from this step were used in the bioassays.

Table 1. Nucleopolyhedrovirus (NPV) isolates obtained from different insect species and evaluated, with their respective source.

Isolated	Insect species	Location
HearNPV-BR1	*Helicoverpa armigera*	Sete Lagoas, Minas Gerais, Brazil
HearNPV-BR2	*Helicoverpa armigera*	Sete Lagoas, Minas Gerais, Brazil
HearNPV-BR3	*Helicoverpa armigera*	Sete Lagoas, Minas Gerais, Brazil
SfNPV- BR4	*Spodoptera frugiperda*	Cascavel, Paraná, Brazil
Gemstar (Gem)	*Helicoverpa zea*	USA, imported

GENETIC IDENTIFICATION

DNA was extracted from all local isolated viruses. This DNA was used in bioassays, and was subjected to genetic sequencing by the Virology Laboratory of the Department of Cell Biology at the Institute of Biological Sciences of the "Universidade de Brasília" in Brasilia, Distrito Federal, Brazil. Dead caterpillars were macerated, filtered 3 times in gauze and cotton, centrifuged at 15,000 rpm for 15 min, washed 4 times with 2% sodium dodecyl sulfate, and rinsed in distilled water for sample purification and to obtain polyhedra for the bioassays. Polyhedral solution (50 µL) was subjected to phenol chloroform extraction for DNA isolation, which was subsequently amplified by PCR and sequenced (Rowley et al. 2010). Highly conserved loci (lef-8 and lef-9) were amplified with degenerate primers lef-8 prL8F2 (forward): 5'GTAAAACGACGGCCAGTNNNACNRCNGARGAYCC3' prL8R2 (reverse): 5'AACAGCTA TGACCATGMMNCCYTTYTGNCCRTG3', and specific for lef-9 prL9–2 (forward): 5'TGTAA AACGACGGCCAGTTTGTCDCCRTCRCARTC3' prL9–1 (reverse): 5'CAGGAAACAGCTAT GACCAARAAYGGITAYGCBG3' (Craveiro et al. 2013). Sequencing with degenerate primers was performed by Macrogen, Geumcheon-gu, Seoul, South Korea. The sequences obtained were submitted to the CAP 3 program for quality verification. Nucleotide sequences from good quality samples were submitted to the Basic Program Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to compare similarity with sequences in the global GenBank repository (http: //www.ncbi.nlm.nih.gov). The sequences were compared using the BLASTN nucleotide database. The results were classified according to the expected E-Value for genes lef-8 (identity > or = 98%) and lef-9 (identity > or = 96%).

The partial DNA sequences of the lef-8 and lef-9 genes were aligned in Clustal W in MEGA 7 version 7.0.14, along with corresponding partial sequences from 10 different baculoviruses, including the control Gemstar (Accession No. HQ246097.1 and HQ246124.1) available from the GenBank database list after alignment. The alignments were trimmed to the size of the partial HearNPV sequences, and the alignments of each gene were concatenated in BioEdit version 7.2.5 (Hall 1999). Maximum Likelihood method analyses were performed in MEGA software version 7.0.14 4 (Tamura et al. 2011). The trees were built by 1,000 replicates of stepwise addition.

BIOLOGICAL ACTIVITY OF NUCLEOPOLYHEDROVIRUS IN *HELICOVERPA ARMIGERA* LARVAE

Six-d-old third instar *H. armigera* larvae were held without food for 4 h, and subsequently received a 1.8 × 1.8 cm disk from a corn leaf impregnated with 20 µL of polyhedral suspension. Viral suspensions were prepared using different dosage of the 5 isolates. These dosages were 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 polyhedra per mL. Tween 80® (50 µl per suspension) was added with the aid of a positive displacement pipette. Dosages of viral isolates were determined in advance using a hemocytometer (Neubauer chamber, Kasvi, São Paulo, São Paulo, Brazil). Larvae that consumed the entire leaf disc in 48 h were transferred to containers with an artificial diet without the pathogen. Each treatment had 32 larvae. Control leaf discs were treated with sterile water and Tween 80®. *Helicoverpa armigera* mortality was recorded every 24 h for 10 d, and nucleopolyhedrovirus infection was confirmed by visualization of the pathogen in the tissues of dead hosts (Rowleya et al. 2011).

The median lethal dose (LD_{50}) was calculated for each isolate using PROBIT regression for 10 d of observation. The average lethal time (LT_{50}) was estimated by Kaplan-Meier survival analysis using a dosage of 1×10^6 polyhedra per mL. This was the lowest dose determined to produce 100% larva mortality in Gemstar® isolates.

Results

GENETIC IDENTIFICATION

The phylogenetic analysis based on concatenated sequence of lef-8 and lef-9 genes, confirmed that HearNPV collected in Brazil is closely related to baculovirus HearNPV of other regions. BR1 (Accession Nos. MF542296 and MF542300), and BR3 (Accession Nos. MF542298 and MF542302), were related to each other and closely related to nucleopolyhedrovirus from Australia (HearNPV-Aus, Accession No. JN584482.1), China (HearNPV-Complete Genome, Accession No. AF303045.2), and HearNPV-G4, (Accession No. AF271059.2). BR2 (Accession Nos. MF542297 and MF542301) was closely related to nucleopolyhedrovirus from South Africa (HearNPV-NNg1, Accession No. AP010907.2), HearNPV-Aus, HearNPV-CI (Accession No. AF303045.2), and HearNPV-G4. BR4 (Accession Nos. MF542299 and MF542303) was not related to any HearNPV, but was closely related to isolated control, HzSNPV-Gemstar (Accession Nos. HQ246097.1 and HQ246124.1), and nucleopolyhedrovirus from India (HearNPV-Faridkot, Accession No. KM357512.1) (Fig. 1).

BIOLOGICAL ACTIVITY OF THE NUCLEOPOLYHEDROVIRUS IN *HELICOVERPA ARMIGERA* LARVAE

The virus isolates caused infection and death in the third instar *H. armigera* larvae within 10 d, with values directly reflecting their dosages. The LD_{50} values were 7.2×10^4, 8.0×10^4, 4.1×10^4, 3.5×10^5, and 5.0×10^5 polyhedra per mL for the isolated Gemstar, BR1, BR2, BR3, and BR4 strains, respectively (Table 2). The lower and upper limits for the LD_{50} differed among the strains, with lowest values for the BR2. The limits for the Gemstar and BR1 isolates showed intermediate values. The BR3 and BR4 isolates showed higher limits, with values above the maximum dose for the other isolates. The BR2 isolate was the most virulent based on assessment of mortality.

The LT_{50} value ranged from 7 to 10 d. With the BR1 and BR2 isolates, survival was 50% at approximately 7 d after inoculation. The survival curve reached LT_{50} at 8, 9, and 10 d for the Gemstar®, BR3, and BR4 isolates, respectively. In the control treatment (no virus), all larvae were alive after 10 d (Fig. 2). The slope of the survival curve showed differences between the isolates in terms of LT_{50} values, with faster replication of BR1 and BR2, resulting in faster host death.

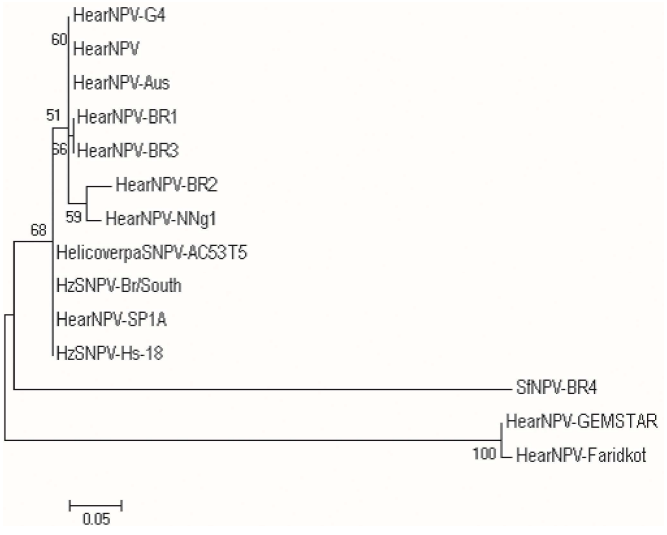

Fig. 1. Phylogenetic relationships among HearNPV isolates (BR1, BR2, BR3, Br4, and GEM) based on analysis of concatenated nucleotide sequences of the lef-8 and lef-9. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (−799.3699) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated.

Discussion

The recent presence of *H. armigera* in Brazil indicates that it may be susceptible to natural enemies (Czepak et al. 2013; Specht et al. 2013; Costa et al. 2015), commonly collected during pest outbreaks, and used for integrated pest management in different regions of the world (Wyckhuys et al. 2013; Luo et al. 2014). Naturally occurring isolates of pathogenic *H. armigera* baculovirus in Brazil could allow for the manufacture of locally produced insecticides without importing viruses (Moscardi 1999; Rollie et al. 2013). The selection of baculoviruses in Spain (Arrizubieta et al. 2013), China (Luo et al. 2014), and Australia (Buerger et al. 2007) allowed for the identification of local *H. armigera* isolates and the subsequent development of biological insecticides.

Genetic identification and biological tests can be used to select virus isolates with high potential for biological control. Genetic variations in baculovirus populations collected in the field can increase performance for biological control (Cory et al. 2005; Baillie & Bouwer 2012). Observed differences in the DNA sequences of isolates show that baculoviruses can be identified, and allows for the characterization of new virus isolates, as reported for those used in China against *H. armigera* (Tang et al. 2012).

The high degree of relatedness among Brazilian *H. armigera* virus isolates and those of Australia (HearNPV-Aus), China (HearNPV-Complete Genome), and South Africa (HearNPV-Nng1) suggests the presence of highly specific baculovirus infecting *H. armigera* in Brazil. HearNPV baculovirus had not been reported previously in this country, and therefore they may have been introduced by *H. armigera* populations. The genetic similarity between Brazilian nucleopolyhedroviruses and those of other countries that often are used in outbreaks of this pest indicates that these microorganisms and their insect hosts can overcome geographical barriers and colonize new habitats (Mazzi & Dorn 2012). The identification of more than 1 baculovirus nucleopolyhedrovirus shows that they were introduced by various means, as reported for *H. armigera* populations in Brazil (Leite et al. 2014). Unlike in the first outbreak of *H. armigera* in Brazil, we found multiple isolates, whereas Ardisson-Araújo et al. (2015) identified only 1 as HzNPV.

The natural dispersion of baculovirus is possible due to their ability to exist in a dormant state during different insect stages (Vilaplana et al. 2010). This occurs when viruses are ingested at sublethal concentrations (virus replication is non-existent), and as a result fail to cause disease signs (Burden et al. 2003; Cory & Myers 2003). The latent virus can be activated and initiate infection, or can be transmitted to the host offspring (Kukan 1999). Stress factors to the hosts, such as suboptimal temperatures and nutrition, high density, and the presence of other pathogens within the same host, can activate the virus (Fuxa

Table 2. Response dose and mortality of third instar *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae with nucleopolyhedrovirus isolates.

Isolate	N	Slope ± SE	X^2	LD_{50}	Range	*P*	df
Gem	192	1.3984 ± 0.2988	8.6068	7.2×10^4	$2.1 \times 10^4 – 2.0 \times 10^5$	< 0.001	4
BR1	192	1.2791 ± 0.1818	4.5663	8.0×10^4	$4.8 \times 10^4 – 1.2 \times 10^5$	< 0.001	4
BR2	192	0.9123 ± 0.1386	4.0808	4.1×10^4	$1.9 \times 10^4 – 7.5 \times 10^4$	< 0.001	4
BR3	192	0.9754 ± 0.1769	8.5379	3.5×10^5	$1.0 \times 10^5 – 1.3 \times 10^6$	< 0.001	4
BR4	192	0.9373 ± 0.1606	8.1627	5.0×10^5	$1.4 \times 10^5 – 1.8 \times 10^6$	< 0.001	4

Isolate = Nucleopolyhedrovirus Isolates; Gem = Gemstar®LC; N = number of assays; Slope ± SE = standard error; X^2 = chi-square; LD_{50} = average lethal dose; Range = Interval between the lowest and the highest value for LD_{50}; *P* = p-value; df = degrees of freedom.

et al. 1999; Takatsuka et al. 2007). In this study, laboratory conditions imposed on *H. armigera* larvae collected in the field could have caused stress, resulting in activation of the BR1, BR2, and BR3 baculovirus isolates, which can pass through insect generations (Kukan 1999). Virus transmission between generations is classified as vertical transmission, and often is reported in baculovirus species (Kukan 1999). Such nucleopolyhedrovirus contamination has been reported in fifth instar *Bombyx mori* (L.) (Lepidoptera: Bombycidae) larvae with adults producing progeny infected with the same isolate as their ancestors (Khurad et al. 2004). Similarly, 30.9 ± 2.9% *H. armigera* progeny were infected due to vertical transmission of virus isolates in laboratory tests in China (Zhou et al. 2005). Latent baculovirus has been reported in 60 to 80% of progeny of the second generation of insects infected with sublethal doses of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) virus (Burden et al. 2002).

The BR4 isolate may have originated from a latent baculovirus activated by the SfNPV virus, or the baculovirus SfNPV may have been modified to adapt to a new host and thereby caused infection in *H. armigera*. For the first possibility, a heterologous virus (isolated from a different species) can trigger infection with homologous virus (isolated from the same species) in a latent state within the host (Matthews et al. 2002). This was demonstrated for a laboratory population of *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae) when a latent baculovirus was activated after infection with an AcNPV virus [*Autographa californica* (Speyer) (Lepidoptera: Noctuidae) nucleopolyhedrovirus isolates that were phylogenetically distant from MbNPV (nucleopolyhedrovirus isolates of *M. brassicae*)] (Hughes et al. 1993). On the other hand, some reports indicated the possibility of baculovirus adapting to new hosts and causing high mortality rates (Jehle et al. 2006). Rabalski et al. (2016) found this to be the case when they evaluated the similarity of the alphabaculovirus isolated from dead *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) larvae and showed high genetic similarity to baculovirus previously isolated from *Lymantria monacha* (L.) (Lepidoptera: Lymantriidae).

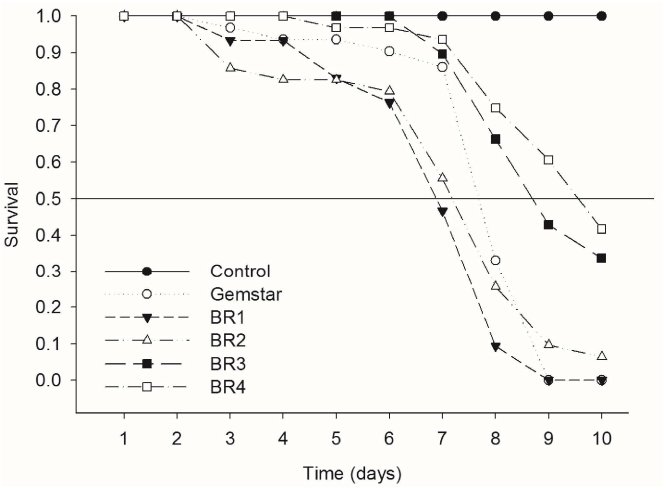

Fig. 2. Survival curves of *Helicoverpa armigera* (Lepidoptera: Noctuidae) after feeding on corn leaves inoculated with 1×10^6 polyhedra per mL of baculovirus isolated. Gemstar®LC, BR1, BR2, BR3, BR4, and in control obtained with Kaplan-Meier estimator.

Virus isolates caused third instar *H. armigera* larval mortality at the evaluated doses. Analysis of LD_{50} values indicated that the BR2 isolate was more virulent as compared with the other isolates. The LD_{50} values were similar to those reported in screening tests for biological pest control programs of *H. armigera* in Spain (Arrizubieta et al. 2013). The average lethal time of 7 d for *H. armigera* is similar to that reported in laboratory tests with third instar larvae infected with different baculovirus isolates (Ogembo et al. 2005; Arrizubieta et al. 2013).

Infection with BR2 resulted in a higher mortality of *H. armigera* larvae, and was thus identified as the most virulent strain, with lower dosage of polyhedra and shorter replication periods required. The faster time to insect pest death induced by virus polyhedra is a key factor in choosing a baculovirus isolate as a biopesticide (Raymond et al. 2006; Kutinkova et al. 2012). Differences in virulence between nucleopolyhedrovirus isolates are in accordance with results of baculovirus strains collected in the Iberian Peninsula on second instar *H. armigera* larvae, which showed that the isolate HearSNPV-SP1 caused more rapid mortality than the other Iberian strains tested (Arrizubieta et al. 2013).

The higher activity of a local isolate against *H. armigera* in Brazil implies greater biological potential of BR2 for biological control programs of this pest. This is the first report of local nucleopolyhedrovirus isolates in *H. armigera* in Brazil. The identification of a baculovirus strain with high specificity to insect larvae from field studies reinforces the existence of dormant baculovirus in the larvae of *H. armigera*.

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