Virus Reviews and Research
Sociedade Brasileira de Virologia
journal homepage: www.vrrjournal.org.br/

Research Article

CHARACTERIZATION OF HELICOVERPA ZEA SINGLE NUCLEOPOLYHEDROVIRUS ISOLATED IN BRAZIL DURING THE FIRST OLD WORLD BOLLWORM (NOCTUIDAE: HELICOVERPA ARMIGERA) NATIONWIDE OUTBREAK

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ABSTRACT

A baculovirus isolated in Brazil during the first nationwide outbreak of *Helicoverpa armigera* is described by ultrastructural analyses, restriction profiles, pathogenicity of host insects, and complete genome sequence. The results revealed that the virus is an isolate of the species *Helicoverpa zea single nucleopolyhedrovirus* (HzSNPV-Brazilian) never reported before in Brazil. Among the HzSNPV isolates few mutations were observed depicting likely a recent divergence of this lineage. Therefore, the entrance of both foreign pests and natural pathogens into the country must warn the government to reinforce sanitary barriers in order to avoid possible agriculture sabotage and novel foreign pest introductions. Moreover, we found that the Brazilian natural isolate was as lethal as a commercial strain to *H. armigera*. Importantly, virus characterization is of importance in establishment of an economical and useful virus-based biological control program in the country to counteract effectively pest infestations.

Keywords: Helicoverpa argimera, pest outbreak, Brazil, baculovirus, HzSNPV, biological control.
Received in April 22, 2015 - Accepted in May 25, 2015 - Published online in May 31, 2015

INTRODUCTION

In February 2013 the old world cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), that used to be restricted to Africa, Asia, and Europe was identified for the first time in Brazil. A month later, the Brazilian Corporation of Agricultural Research (Portuguese acronym EMBRAPA) reported this occurrence to the Brazilian Ministry of Agriculture, Livestock, and Food Supply (Notification Report n° 70570.000355/2013-2) (Specht et al. 2013). Unfortunately, by that time the crop pest was already spread in a high prevalence in the country, which has led to severe agriculture damages and economical losses. This outbreak could be explained by an association of both inadequate management of planting host species (*e.g.* cotton, soybean, and corn) in extensive areas and the uncontrolled use of chemical pesticides which provided together optimal conditions for insect growing.

The genus *Helicoverpa* presents some of the most devastating pest species in the world causing hefty economic losses in several crops including cotton, soybean, wheat, corn, green beans, tomatoes, citrus, and pastures (Cunningham & Zalucki, 2014). The larvae are naturally more tolerant to most of the common insecticides requiring higher application rates to be controlled efficiently (McCaffery, 1998). Almost 30% of all pesticides used worldwide are directed against *H. armigera* (Ahmad, 2007) although the management of outbreaks has so far been ineffective and also has induced the appearance of resistant insect phenotypes (Oakeshott et al. 2013; Rowley et al. 2011) including engineered plants expressing *Bacillus thuringiensis* (Bt) toxins (Alvi et al. 2012). Therefore, other naturally found disease-causing pathogens like baculoviruses are important alternatives for the integrated and effective control of *Helicoverpa* (Rowley et al. 2011). Robust virus characterization allows the establishment of a virus-based biological control program to control pest outbreaks as a safety, useful, and economical alternative for chemical pesticides.

For the crop season 2013/2014, commercial baculoviruses infective to the old world bollworm have been imported to be used in Brazil. Before this allowance by the Brazilian government to import *Helicoverpa*-infecting baculoviruses from other countries in order to control a nationwide *H. armigera* outbreak, a baculovirus was isolated in field from larvae cadavers with symptoms of infection. Cadavers of *H. armigera* were collected in March/2013 on soybean crops in Warta, Londrina County, Parana, Brazil. Although *H. zea* does not infest soybean in Brazil, we confirmed the species *H. armigera* by amplifying and sequencing the genes *cytochrome c*

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doi - http://10.17525/vrrjournal.v20i1.254

oxidase I (COI), *cytochrome B*, and the region *cox1-tRNA-leu-cox2* (data not shown). Electron microscopy (EM) of purified occlusion bodies (OBs), which are hallmarks of the family *Baculoviridae*, showed polyhedral shape (FIG. 1A) and virions with singly enveloped nucleocapsids within (FIG. 1B). The occlusion bodies purification, polyhedra EM and DNA extraction were performed according to published protocols (Ardisson-Araujo et al. 2014). The viral DNA (1–2 µg) was individually cleaved with the restriction enzymes XhoI, BglII, PstI, or BamHI (Promega) according to manufacturer's instructions. Importantly, HzSNPV is found naturally infecting the genus *Helicoverpa* during its larval stage (Chen et al. 2002; Ogembo et al. 2009; Rowley et al. 2011). Based on the comparison of both the viral DNA restriction enzyme profiles (FIG. 1C) and previously published data of other *Helicoverpa*-infecting nucleopolyhedroviruses (Chen et al. 2002), we concluded that the virus belonged to the species HzSNPV which was one of the first commercial baculovirus pesticides registered in the 1970's (Virion-H, Biocontrol-VHZ, Elcar) and has been so far produced and applied successfully against both *H. armigera* and *H. zea* (Rowley et al. 2011; Shieh, 1989; van Beek & Davis, 2007). Therefore, we named the Brazilian isolate HzSNPV-Brazilian, even being found in *H. armigera* cadavers.

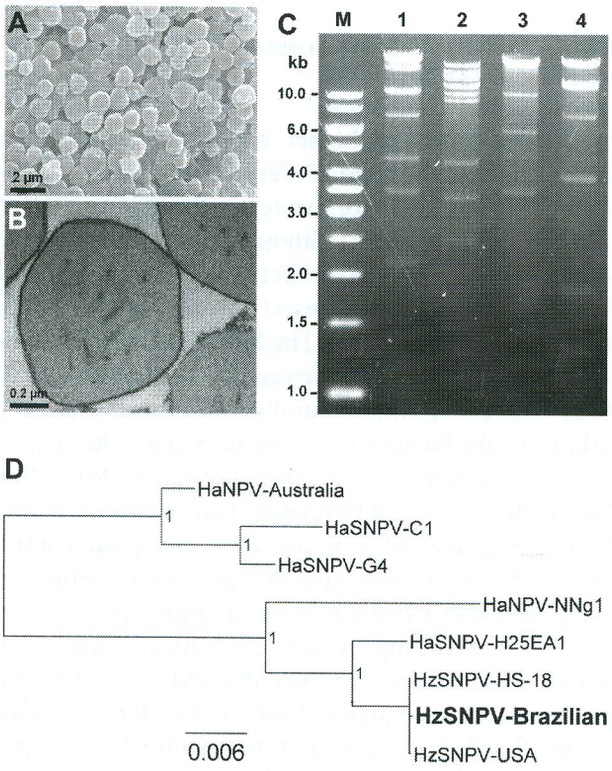

Figure 1. Characterization of the Helicoverpa-infecting baculovirus found in Brazil. (A) Scanning electron micrograph shows polyhedral-shaped OBs. (B) Transmission electron micrograph shows sliced OBs with single-enveloped nucleocapsids within. (C) Agarose gel electrophoresis-resolved HzSNPV-Brazilian genome DNA fragments digested with XhoI (lane 1), BglII (lane 2), PstI (lane 3), and BamHI (lane 4), and molecular weight marker (lane M). All the features together corroborate that this isolate belongs to the species Helicoverpa zea single nucleopolyhedrovirus (HzSNPV). (D) Maximum likelihood tree of Helicoverpa-isolated single nucleopolyhedroviruses. The phylogeny was inferred using MAFFT alignment of whole genome and the relationship using PhyML method. The Brazilian isolate (boldface) is related to both HzSNPV-USA and HzSNPV-HS18 viruses and the closest relative to this group is the Australian HaNPV-H25EA1. Branch support is estimated by a Shimodaira–Hasegawa-like test.

To further substantiate our data, we carried out a bioassay using the Brazilian strain and a commercially available virus from the same species HzSNPV (Gemstar®) towards *H. armigera* and *H. zea*. For this experiment, serial dilution of the virus were carried out to determine both LC_{50} and LC_{99} in third-instar caterpillars and mixed with the larva diet as previously described (Ardisson-Araujo et al. 2014). Insects were allowed to feed *ad libitum* on virus inoculated diet. A group with no treatment (n=60) was set up as control. Mortality was recorded 13 days post-infection (p.i.) by scoring the number of dead insect which had no response to touch. The data was analyzed by Polo Plus program (LEORA SOFTWARE, POLO-Plus 1.0, Probit and Logit analysis, Petaluma, California. 2003). We found that the Brazilian isolate virus was more lethal to *H. zea* than to *H. armigera* in oral bioassays (Table 1). The OB concentration per ml of artificial diet capable to kill 50% of the tested insects at the third-instar (LC50) was 987 OB/ml to *H. armigera* and 215 OB/ml to *H. zea*. This ability to kill *H. zea* more efficiently by HzSNPV was previously reported (Rowley at al. 2011), which is a very interesting aspect of short term adaptation to the host even presenting high identity to the closest relatives (*i.e.* HaNPV isolates). Moreover, we tested whether the Brazilian strain could be as efficient as the commercially available HzSNPV from Gemstar® (Certis, Columbia, USA) to kill *H. armigera*. We found that both viruses had statistically equal lethal concentration to the tested insect (Table 1). Conversely, in a worldwide *Helicoverpa*-isolated baculovirus study, Gemstar® isolate of HzSNPV presented lethal concentration higher than the other naturally found isolates (Rowley et al. 2011).

The whole genome of HzSNPV-Brazilian (Genbank: KM596835) was sequenced with the 454 Genome Sequencer (GS) FLX™ Standard (Roche) at the Center of High-performance Genomic (Brasilia, Brazil). The genome was *de novo* assembled using Geneious 6.0 (Kearse et al. 2012) and confirmed with the digestion profile. Annotation was also performed using Geneious 6.0 to identify the open reading frames (ORFs) that started with a methionine codon (ATG) encoding polypeptides with at least 50 amino acids, and BLASTP (Altschul et al. 1997) to identify homologs. The sequencing produced 8,237 single-end reads. After size and quality trimming, 8,068 reads (average size of 755.5 nt) were assembled with coverage of 47.2±12.0 bp/site. The HzSNPV-Brazilian

genome has a size of 129,694 bp with a G+C content of 39.1 %. The genome potentially codes for 146 putative ORFs with predicted polypeptides of at least 50 amino acids and all of them are homologs to those of HzSNPV isolates. Eight ORFs were not annotated in the first described genome but were present. Isolates of HzSNPV have a nucleotide pairwise alignment identity of 99% and the average identity across the *Helicoverpa*-infecting SNPVs is 96.22±1.49%. HzSNPV-Brazilian presents a deletion of 1,000 bp in the homolog region 1 (confirmed by PCR, data not shown).

Table 1. Dose-mortality responses of *Helicoverpa* spp. third instar larvae infected orally with either HzSNPV-Brazilian (Br) or a commercial strain of HzSNPV (Gemstar®).

Insect	Virus	n[1]	LC_{50} (OB/ml)	95% Fiducial limits		LC_{99} (OB/ml)
				Lower	Upper	
H. zea	Br	197	2.15×10^2	0.75×10^2	4.00×10^2	130.0×10^2
H. armigera	Br	482	9.87×10^2	6.60×10^2	15.6×10^2	754.0×10^2
	Gemstar®	283	10.2×10^2	4.71×10^2	21.5×10^2	nt

[1], number of tested insects; nt, non-tested

For phylogenetic analysis, a MAFFT alignment (Katoh et al. 2002) was carried out with whole genome sequences of all *Helicoverpa*-isolate single nucleopolyhedrovirus available in Genbank. This alignment was manually inspected, and poorly aligned regions (at least 50 % of gaps) were deleted. The resulting alignment was approximately 135 kb long. The maximum likelihood tree was inferred using PhyML (Guindon et al. 2010), under Tamura-Nei model selected by jModelTest-2.1.4 software (Darriba et al. 2012). The branch support was estimated by a Shimodaira-Hasegawa-like test (Anisimova et al. 2011). The phylogenetic analysis confirmed that HzSNPV-Brazilian is closely related to HzSNPV isolates (FIG. 1D). The short branch length compared to the other isolates indicates low genetic diversity and low branch support prevented us to establish the origin of the Brazilian strain.

In order to determine the CDS diversity among the *Helicoverpa*-infecting single nucleopolyhedrovirus, we considered the completely sequenced viruses as two separated groups including viruses isolated from (*i*) *H. armigera* and (*ii*) from *H. zea*. To search for polymorphism, we concatenated 135 ORFs found to be common among all the *Helicoverpa*-isolated single nucleopolyhedrovirus: HaNPV isolates C1 [AF303045], Australia [JN584482], G4 [AF271059], NNg1 [AP010907], and H25A1 [KJ922128] and HzSNPV isolates USA [AF334030], HS18 [KJ004000], and Brazilian [KM596835]. We performed a MAFFT alignment and set as reference sequence the genome of the G4 for the HaNPV group and the Brazilian for HzSNPV group. For the first (*i.e.* HaNPV-related baculovirus), we found 624 nonsynonymous polymorphisms out of 1,592 (data not shown). On the other hand, we found only 13 nonsynonymous polymorphisms out of 15 among the three HzSNPV isolates (data not shown). This very low genetic diversity among the HzSNPV isolates in comparison to HaNPV depicts a recent divergence of the isolates reinforcing the hypothesis that the Brazilian isolate could be recently introduced into the country from either the American or the Russian strain. Sublethal and latent infections are of importance for the persistence of baculoviruses in the environment (Kukan, 1999) which could explain how HzSNPV together with the host insect has gotten into the country. In a previous study, we found the first non-Asian isolate of a *Bomby mori*-infecting baculovirus in Brazil. By complete genome sequencing and phylogenetic analysis, similarly to the results found in this work, we found that the virus was probably introduced together with the insect into the country (Ardisson-Araujo et al. 2014).

We determined the following from the present short report. (*i*) The *H. armigera*-infecting baculovirus isolated in Brazil belongs to the species HzSNPV. (*ii*) It is a single NPV with polyhedral-shaped occlusion bodies. (*iii*) The virus was more lethal to *H. zea* than to *H. armigera*, besides of presenting the same lethality as that observed for the commercial strain Gemstar® to *H. armigera*. (*iv*) The complete genome sequence revealed its close relationship to HzSNPV isolates. (*v*) Low genetic diversity was observed among the HzSNPV isolates.

ACKNOWLEDGEMENTS

We thank 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq), 'Fundação de Apoio à Pesquisa do Distrito Federal' (FAPDF), and 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior' (CAPES) for financial support; Ingrid Gracielle Martins da Silva for kindly helping with the sample for TEM and SEM.

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