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Characterization of a *Chrysodeixis includens nucleopolyhedrovirus* Isolate from Brazilian Cerrado and Assessment of its Co-Infection with *Anticarsia gemmatalis multiple nucleopolyhedrovirus*

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HIGHLIGHTS

- ChinNPV-Buritis is a potential isolate for the development of biopesticide.
- Its biological and molecular features are similar to other ChinNPV isolates.
- *In vitro* infection in SF21, Sf9, and Tn-5B1-4 cell lines presented few OBs.
- ChinNPV and AgMNPV co-infected Tn-5B1-4 cells *in vitro*.

Abstract: *Chrysodeixis includens* has become the major Lepidopteran pest of soybean crops, especially in the Brazilian Cerrado (savanna) region. A native isolate of *Chrysodeixis includens nucleopolyhedrovirus* (ChinNPV) from this region, Buritis, MG, was assessed for its biological and molecular features. In addition, *in vitro* co-infection with *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV), another virus of an important soybean pest, was tested. The ChinNPV-Buritis isolate presented an average LC_{50} of 7,750 occlusion bodies (OBs)/ml of diet in *C. includens* larvae. Analysis of restriction endonuclease profiles of viral DNA revealed similarities with previously described ChinNPV isolates IE, IF, and IG from Brazil, although the presence of submolar bands indicates genetic heterogeneity. Optical microscopy analysis in conjunction with quantitative PCR (qPCR) demonstrated *in vitro* infection of this isolate in IPLB-SF-21AE, Sf9, and BTI-Tn-5B1-4 cell lines, but the amount of ChinNPV tends to decrease through serial passages. The qPCR method developed in this study successfully detected both AgMNPV and ChinNPV from cell culture and from infected larvae. The cell line Tn-5B1-4 is indicated for future development of *in vitro* production and co-infection studies.

Keywords: AgMNPV; baculovirus; ChinNPV; soybean looper; velvetbean caterpillar.

INTRODUCTION

In the last few seasons, severe infestations of Lepidoptera larvae has occurred in the large Brazilian savanna region known as the Cerrado. The increase of larvae populations was due to the large areas for growing crops (soybean and cotton) and the intense use of fungicides to control Asian soybean rust, which reduces the entomopathogenic fungi inoculum (*Metarhizium rileyi*, *Zoophthora radicans*, *Pandora gammae*, *Isaria tenuipes*)[1]. In soybean, the most important defoliators are *Chrysodeixis includens* and *Anticarsia gemmatalis*. However, other species, such as *Helicoverpa armigera* and *Spodoptera* (*S. cosmioides*, *S. eridania*, *S. albula,* and *S. frugiperda*) have also been reported, although with much lower prevalence and in restricted areas[2].

Control failures and pest resurgence have triggered the demand for biological control agents. The use of these agents is highly desirable due to their ability to be harmonized in Integrated Pest Management Programs (IPM). In this respect, baculovirus biopesticides are specific and natural control agents of their hosts. Viruses in the family *Baculoviridae* are host specific, infecting only one or a few closely related species of insects. The specificity is innately linked to the pathogenesis of this family of viruses. The occlusion derived virions (ODVs) that emerge from occlusion bodies (OBs) are the universal virion phenotype for all baculoviruses as they are responsible for the initial oral (*per os*) infection of host insect gut cells. In lepidopteran hosts, the initial, primary infection of midgut cells by ODVs is followed by secondary infection of tissues within the insect hemocoel that is affected by the budded virion (BV) phenotype. Dependence on the host-cell molecular machinery is reduced over the course of the infection as baculovirus gene expression and regulatory proteins take over. However, host and/or tissue-specific interactions continue to play a role as the infection progresses within the infected host, which will determine whether a patent infection will occur[3].

The two most important soybean lepidopteran pests in Brazil can be infected by their specific baculoviruses such as *Chrysodeixis includens nucleopolyhedrovirus* (ChinNPV) and *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV)[4,5]. Mixed infestations of soybean looper and velvetbean caterpillar in soybean fields have been controlled with conventional insecticides. AgMNPV has been widely used for microbial control of *A. gemmatalis* in Brazil, but its use has decreased since 2003-04 season, while *C. includens* populations became more common. Another factor that affected AgMNPV use has been the reluctance of companies to produce the virus in a competitive market with widespread adoption of Bt soybean, that had the same target pests[1].

The limiting factors for the use of ChinNPV compared to AgMNPV include disruption of larvae integument, an undesirable characteristic for in vivo production, lower virulence than AgMNPV, and restraints on soybean looper mass rearing due to endogamy and colony depletion[1]. A ChinNPV was isolated from *C. includens* in the region of Buritis - Minas Gerais State, located in the Brazilian Cerrado, in 2014. Natural epizootics were reported in *C. includens* larvae infesting soybean crops in that area, promoting mortality in about 30% of the larvae, which reveals the potential of this virus to be used as a biopesticide[5].

In vitro production could potentially overcome the difficulties of large-scale production of baculovirus biopesticides[6]. Therefore, insect cell cultures are interesting systems that support viral replication[7,8,9]. However, due to several technical limitations and prohibitive costs of production, baculovirus *in vitro* production is still unpractical on the commercial scale[9].

Co-infection of two different viral genotypes can result in co-occlusion phenotype[10,11,12]. *In vitro* co-infection studies based on the recombinant *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) lacking the polyhedrin gene and the wild-type AcMNPV resulted in co-occluded virus[10,13]. In addition, Arrizubieta et al.[12] studied *in vivo* co-infection with different genotypes of *Helicoverpa armigera single nucleopolyhedrovirus* (HearSNPV) in *H. armigera* larvae. After five *in vivo* passages of the co-occluded virus, the amount of one genotype increased and the resulting occlusion body (OB) became more virulent and pathogenic. This present work aimed to evaluate biological and molecular features of ChinNPV, obtained from Buritis, and its *in vitro* replication in insect cell lines. In addition, *in vitro* co-infection of AgMNPV and ChinNPV was tested, as well as the methods to assess the proportions of each virus produced in cells and larvae.

MATERIAL AND METHODS

Insect colony and virus stock

Colonies of *A. gemmatalis* and *C. includens* were maintained at 26 ± 2 °C, 70% relative humidity (RH), and 13 h photophase. Larvae were reared as described by Hoffmann-Campo et al.[14] on an artificial diet[15]. All bioassays were conducted on third instar larvae under the same rearing conditions, 26 ± 1.5 °C, 14 h photophase, and 75% RH. *A. gemmatalis* larvae were infected with AgMNPV-2d[16] and *C. includens* infected with ChinNPV-Buritis isolate[5]. The virus isolates were incorporated into the artificial diet (concentration 10^7 OBs/ml) to produce a virus stock for further experiments.

Occlusion body purification and DNA extraction

Dead larvae were kept in the freezer and later used for occlusion body (OB) purification. Insect cadavers were homogenized with buffer (1% ascorbic acid; 2% SDS; 0.01M Tris pH 7.8; 0.001M EDTA pH 8.0), filtered through three layers of cheesecloth, and centrifuged at 7,000 × g for 10 min. The pellet was suspended in TE buffer (0.01M Tris-HCl pH 8.0 and 0.001M EDTA pH 8.0) and again centrifuged at 7,000 × g for 10 min. The dilution and centrifugation steps were repeated four times. The pellet was loaded onto a continuous 1.17 to 1.30 g/ml sucrose gradient, and centrifuged at 104,000 × g for 40 min at 4 °C. The OB band was collected, diluted 4-fold in TE buffer, and centrifuged at 7,000 × g for 15 min at 4 °C[17]. The OB concentration in the suspension was determined using a Neubauer chamber under a microscope (400×). Purified occlusion bodies (10^9 OBs/ml) were dissolved in an alkaline solution (0.1M sodium carbonate) and used to extract DNA according to O'Reilly et al.[18].

Viral genomic DNA restriction analyses

The viral DNA (1–2 µg) of ChinNPV-Buritis was individually cleaved with the restriction enzymes *Hind*III, *Eco*RI, and *Bam*HI (Invitrogen) according to the manufacturer's instructions. The generated DNA fragments were subjected to electrophoresis in 0.8% agarose gel[19]. The restriction endonuclease profile of Buritis isolate was compared to the DNA profiles of three ChinNPV isolates analyzed by Alexandre et al.[20]. The most similar profiles were submitted to *in silico* restriction analysis based on sequences published by Craveiro et al.[21] (GenBank accession numbers: NC026268, KU669293, KU669294) using Geneious 7.1 software. Each sequence was digested *in silico* with *Hind*III, *Eco*RI, and *Bam*HI restriction enzymes and separated on a virtual agarose gel for comparison.

Biological activity of ChinNPV-Buritis isolate

Two bioassays were performed according to Morales and Moscardi[22]. Viral OBs were incorporated into the artificial diet and offered to third instar *C. includens* larvae. Six concentrations were used: 80, 220, 605, 1670, 4580, and 12580 OBs/ml of diet (Greene et al.)[15]. For each treatment, 60–62 larvae were tested. For negative control, the virus suspension was not incorporated to the artificial diet. Larvae were maintained in a B.O.D incubator at a temperature of 26 ± 1.5 °C and 14 h photophase. For mortality analysis, daily observations were made for 15 days. At the end of the trials, the mortality data were corrected using the Abbott's formula[23] and analyzed by probit analysis to determine the lethal concentration (LC_{50}) values with respective 95% fiducial limits (CL)[24]. Analysis was conducted using the Polo Plus Version 2.0 software (LeOra Software).

Cell culture

Trichoplusia ni BTI-Tn-5B1-4[25], also known as High Five cells, *Spodoptera frugiperda* IPLB-SF-21AE[26] and Sf9[27] were used for *in vitro* assays. The cell lines were maintained at 27 °C in TNMFH [Grace's insect medium (Gibco-BRL) supplemented with lactalbumin hydrolysate and yeastolate] containing 10% heat-inactivated bovine fetal serum.

In vitro assays and DNA extraction

The cells were seeded at a density of 1×10^6 per $60mm^2$ well. The viruses were obtained through hemolymph from infected larvae at 4 d.p.i. (days post infection) and allowed to adsorb into cells for 1 hour (passage zero - P0). Infected cells were kept in TNMFH complete medium at 27 °C. Morphological analysis was initially monitored by light microscopy for five days. Then, the supernatants (100 µl) were collected for new infections (P1) using the same procedure. At 5 d.p.i., the supernatants were collected for the second passage (P2). The morphology from P1 and P2 passages were also monitored by light microscopy. Aliquots of supernatant of each passage (100 µl) were collected for DNA purification and real-time PCR (qPCR) tests. The experiment was performed twice. The OBs obtained in the passages were collected, and the concentration was determined using a Neubauer hemocytometer under phase contrast microscopy at 400×. The OBs suspensions (100 µl) were dissolved in an alkaline solution and used to extract DNA with DNeasy Blood & Tissue (Qiagen) following the manufacturer's instructions and then for qPCR tests in duplicates. For co-infection assays, the same amount of AgMNPV and ChinNPV infected hemolymph (100 µl each) per well was used and allowed to adsorb into cells in the P0 infection. During P1 and P2 passages, the same procedure of the single infection was applied.

Real-time PCR (qPCR) standardization

Specific primers were designed for distinct genes of AgMNPV[28] and ChinNPV[21] based on Genbank sequences database. The primer pair gp64FW (5'TAACGGGGGTGTCATCAACG 3') / gp64RV (5'GTTTGCGCCTAATACAGCGG 3') was designed for *gp64* of AgMNPV. The primer pair PholyFW (5'CACCAAGAAAGGAGGCGTGA3') / PholyR (5'ACAGTCGTCGAGCATCACAA 3') was designed for *photolyase* of ChinNPV. The qPCR reaction was made with Rotor Gene SYBR Green Master Mix (Qiagen) using 0.2 µM of each primer and 1 µl of DNA (1 ng/µl), following the manufacturer's instructions. The assays were done in a Rotor gene 5plex HRM platform (Qiagen). Samples were subjected to the following conditions: 95 °C for 5 min; 40 cycles of 95 °C for 5 sec and 60 °C for 10 sec. A melting curve analysis was performed to assure that a homogenous amplification product had been produced. The qPCR products were visualized by electrophoresis on 1% agarose gel in TAE. To determine the standard curve of the assays, a series of 10-fold dilutions (ranging from 1 to 10^{-10}) of viral DNA was tested in triplicate. Dilution 1 corresponds to 10 ng/µl for both viruses. The DNA concentration was checked using a Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific) and compared to low DNA mass ladder (Invitrogen) in 0.8% agarose gel electrophoresis.

The presence of false-positives results was tested with DNA obtained from the larvae and cell culture controls without viruses. In addition, the possible detection of non-target virus species was verified. To validate the detection parameters previously determined in the standard curve, the following dilutions of OBs were performed: (mix 1)- 1×10^6 OBs/µl ChinNPV and 5 OBs/µl AgMNPV; (mix 2)- 5×10^5 OBs/µl AgMNPV and 10 OBs/µl ChinNPV; (mix 3)- 10^3 OBs/µl of each virus. The larvae, cell culture and OBs mixtures were submitted to DNA extraction with DNeasy Blood & Tissue (Qiagen) following the manufacturer's instructions and then to qPCR tests in duplicate.

Biological activity of OBs obtained from *in vitro* co-infection

OBs obtained from *in vitro* AgMNPV and ChinNPV co-infection were collected to infect *A. gemmatalis* and *C. includens* larvae. Three bioassays were conducted using different OBs concentrations (Table 1).

Table 1. Bioassays performed to analyze the biological activity of OBs obtained from *in vitro* AgMNPV and ChinNPV co-infection.

Bioassay – Inoculum	OB concentration	Number of larvae
B1 – *in vitro* P0 passage	10^2 OBs/ml	100 *A. gemmatalis* 100 *C. includens*
B2 – OBs from infected *A. gemmatalis* (B1 assay)	-	24 *C. includens*
B3 – *in vitro* P0 passage	10^7 OBs/ml	40 *A. gemmatalis* 40 *C. includens*

In the first bioassay (B1), an OB suspension (10^2 OBs/ml) from P0 passage of co-infected cells was prepared. Soybean leaf disks (1 cm) were inoculated with 10 µl of the OB suspension (estimated to contain one OB). Each leaf disk was offered to a single larva. The bioassay was conducted with 100 *C. includens* larvae and 100 *A. gemmatalis* larvae. On the second day, a leaf disk was replaced by artificial diet until 7 d.p.i.

For the second bioassay (B2), the OBs obtained from dead *A. gemmatalis* of B1 were used as inoculum. A suspension was prepared with macerated larvae in distilled water (1:10 proportion). Soybean leaf disks (1 cm) were inoculated with 10 µl of the suspension/leaf disk. Each leaf disk was offered to a single larva. The bioassay was conducted with 24 *C. includens* larvae in the same conditions as the first bioassay.

In the third bioassay (B3), an OB suspension (10^7 OBs/ml) from P0 passage of co-infected cells was prepared. Slices of artificial diet (1 cm) were inoculated with 5 µl of the OB suspension. Each slice was offered to a single larva. The bioassay was conducted with 40 *C. includens* larvae and 40 *A. gemmatalis* larvae until 7 d.p.i. For all bioassays, the dead or symptomatic larvae (polyhedrosis disease) were individually collected for DNA extraction and qPCR as previously described. In addition, a control treatment without virus was conducted in the same conditions, using same number of larvae for each species.

RESULTS

DNA restriction profile and biological activity of ChinNPV-Buritis

Characterization of the ChinNPV isolated from Buritis-MG (Brazil) was done by comparing its restriction endonuclease DNA profile to other ChinNPV isolates described by Alexandre et al.[20]. A similar profile was observed with the presence of more than 20 fragments in the digestions with *Bam*HI, *Eco*RI, *Hind*III, and presence of submolar bands, indicating this isolate might be a mixture of different genotypes (Fig. 1). Since the restriction endonuclease profile resembles that observed in IE, IF, and IG isolates (collected at

Iguaraçu-PR, Dourados-MS, and Sertanópolis-PR, Brazil, respectively), they were chosen for *in silico* DNA endonuclease digestion for more detailed comparison. The analysis revealed a distinct *Eco*RI profile of ChinNPV-Buritis, presenting the fragment of 15 kb observed for the IF and IG and the fragments of 11 kb and 3.6 kb observed for IE. The *in silico Eco*RI digestion analysis for isolates IF and IG differed from that performed with viral DNA by Alexandre et al.[20], which have the fragments of 11 kb and 3.6 kb. Virulence assays to its host (*C. includens*) found that ChinNPV-Buritis isolate presented average LC_{50} of 7,750 OB/ml (Table 2), which is an intermediate value between the LC_{50} of the isolates, IE/F and IG. The IE and IF isolates presented the lowest LC_{50} among the six Brazilian isolates compared by Alexandre et al.[20]. However, the IG had the highest LC_{50} among these isolates.

Table 2. Lethal concentration (LC_{50}) of ChinNPV-Buritis isolate ($OB \times mL^{-1}$) incorporated into the artificial diet and offered to third instar *C. includens* larvae

Treatment	Days p.i.	N[1]	LC_{50}	CI (95%)	Slope ± (SE[2])	df	χ^2
First	15	432	8792	5602- 16752	1.031 ± 0.179	4	3.712
Second	15	417	6709	3834- 14110	1.736 ± 0.285	4	4.3673

N[1]= number of insects tested
SE[2]= Standard error

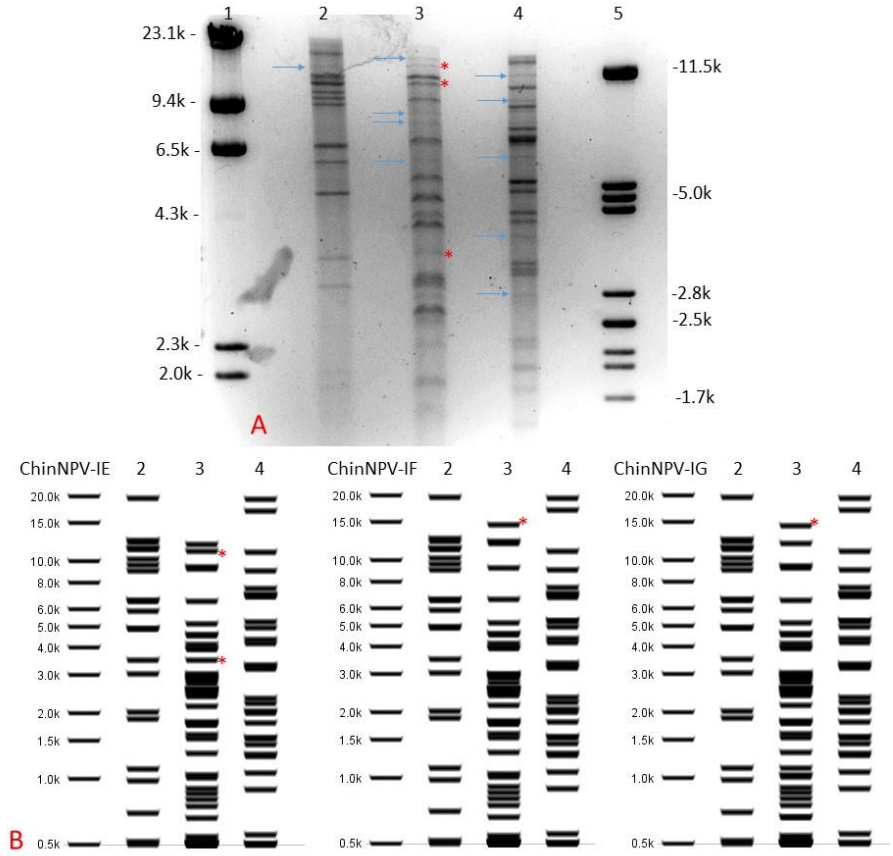

Figure 1. Restriction endonuclease profile of ChinNPV-Buritis DNA (A) and *in silico* restriction endonuclease profile of ChinNPV-IE, ChinNPV-IF, and ChinNPV-IG based on DNA sequences available on GenBank (B). ChinNPV DNA was digested with *Bam*HI (2), *Eco*RI (3), and *Hind*III (4). Molecular marker DNA ʎ *HindIII* (1) and DNA ʎ *Pst*I (5). Restriction fragments were separated by electrophoresis in 0.8% agarose gel. Arrowed fragments are submolar bands present in ChinNPV-Buritis, and asterisks indicate bands unique to isolates IE, IF, and IG present in ChinNPV-Buritis.

Analysis of specificity, sensibility, and reproducibility of Real-time qPCR

The primers designed for the qPCR efficiently detected the viruses. The healthy larvae and cell culture controls did not show amplification. There were no false-positive results for non-target viruses. The melting curve analysis showed a single peak for both primer pairs, indicating high specificity. The amplicons visualized after electrophoresis analysis presented the expected size, while non-specific products were not amplified. For the primer pair gp64FW/RV, the standard curve exhibited the equation of $y = -3.596x + 15.250$ and the coefficient of correlation (R^2) of 0.997. It displayed an amplification efficiency of 90%. For the primer pair PholyFW/PholyRV, the standard curve showed the equation of $y = -3.507x + 13.852$ and the coefficient of correlation (R^2) of 0.998. It displayed an amplification efficiency of 93%.

The detection limit of both reactions was 10^{-5} ng DNA, which corresponded to two OBs of AgMNPV and four OBs of ChinNPV in this assay. The validation of detection parameters presented minimal variation in the technical duplicate and in the assays repeated in time (Table 3), demonstrating the reproducibility of the technique. This variation should be considered normal due to the different number of virions in each OB, as well as the intrinsic characteristic of nucleic acid extraction methods[29,30].

Table 3. Real-time qPCR quantification of *photolyase* and *gp64* genes in DNA extracted from mixtures of AgMNPV and ChinNPV occlusion bodies

Mixtures	Viral DNA (ng/µl)	
	ChinNPV – *photolyase*	**AgMNPV- *gp64***
Experiment 1		
1×10^6 OBs/µl ChinNPV + 5 OBs/µl AgMNPV	9.12	1.67×10^{-3}
5×10^5 OBs/µl AgMNPV+ 10 OBs/µl ChinNPV	1.79×10^{-4}	16.0
10^3 OBs/µl of AgMPNV+ 10^3 OBs/µl ChinNPV	2.79×10^{-2}	6.22×10^{-2}
Experiment 2		
1×10^6 OBs/µl ChinNPV + 5 OBs/µl AgMNPV	7.05	2.08×10^{-3}
5×10^5 OBs/µl AgMNPV+ 10 OBs/µl ChinNPV	6.15×10^{-4}	12.6
10^3 OBs/µl of AgMPNV+ 10^3 OBs/µl ChinNPV	3.90×10^{-2}	5.55×10^{-2}

In vitro assays

Single infection of ChinNPV in SF21, Sf9, and Tn-5B1-4 cell lines resulted in low OBs production. Cell culture visualization by optical microscopy (Fig. 2), in conjunction with the qPCR assays of cell culture supernatants (Table 4), indicates that infection with ChinNPV is restricted to individual cells. The best OB production occurred in Tn-5B1-4 cell lines. In SF21 and Sf9 cell lines, OBs production was observed only in a few cells.

The results confirmed that ChinNPV viral DNA concentration decreases over passage even in the presence of AgMNPV. However, the amount of AgMNPV tends to increase in the first passage and to stabilize in the second passage (Table 4). Both viruses were also detected in the qPCR test from OBs collected from *in vitro* co-infections, although the amount of ChinNPV was low compared to AgMNPV (Table 5).

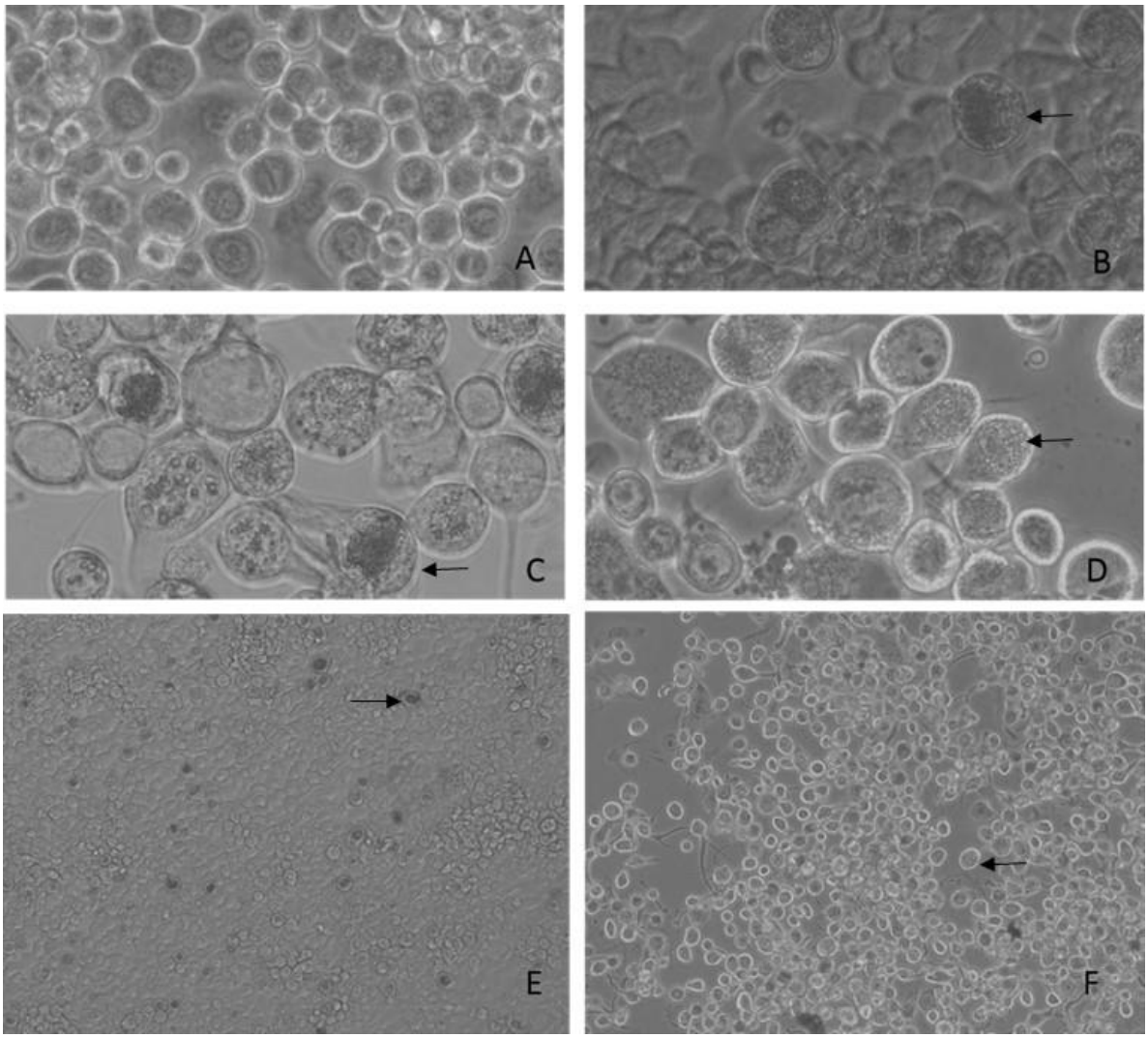

Figure 2. Phase-contrast micrographs of *T. ni* BTI-Tn 5B1-4 cells - P0 passage at 96 h p.i. Mock-infected cells (A). Cells infected with ChinNPV-Buritis (B). Cells co-infected with ChinNPV-Buritis and AgMNPV-2d (C). Cells infected with AgMNPV-2d (D) (20× objective). Cells infected with ChinNPV-Buritis (E) and cells co-infected with ChinNPV-Buritis and AgMNPV-2d (F) at 10×objective. The arrows show the OBs and cytopathic effects in the cells.

Table 4. Real-time qPCR quantification of *photolyase* and *gp64* genes in DNA extracted from cell culture supernatant infected with ChinNPV and co-infected with AgMNPV and ChinNPV.

Passage	**Cell line**	**ChinNPV infection**	**ChinNPV+AgMNPV co-infection**	
		ChinNPV *photolyase* (ng/µl)	**ChinNPV *photolyase* (ng/µl)**	**AgMNPV *gp64* (ng/µl)**
Experiment 1				
P0	Sf21	NT*	1.2×10^{-4}	0.11
	Sf9	NT	2.8×10^{-4}	6.2×10^{-2}
	Tn5B1-4	2.4×10^{-3}	1×10^{-4}	9.6×10^{-3}
P1	Sf21	4.2×10^{-4}	2.5×10^{-5}	1.1
	Sf9	2.8×10^{-4}	1.7×10^{-5}	0.3
	Tn5B1-4	2.8×10^{-4}	1.3×10^{-5}	0.32
P2	Sf21	1.2×10^{-5}	ND	0.23
	Sf9	4×10^{-6}	ND	0.17
	Tn5B1-4	6.3×10^{-6}	ND	7.5×10^{-2}
Experiment 2				
P0	Sf21	NT	7.7×10^{-6}	1.3×10^{-2}
	Sf9	NT	8.2×10^{-6}	5.4×10^{-3}
	Tn5B1-4	9.9×10^{-5}	5.2×10^{-6}	2.3×10^{-2}
P1	Sf21	1.3×10^{-5}	ND	7.5×10^{-2}
	Sf9	1×10^{-5}	ND	2.7×10^{-2}
	Tn5B1-4	3×10^{-6}	ND	4.7×10^{-2}
P2	Sf21	ND*	ND	3.7×10^{-2}
	Sf9	ND	ND	2.3×10^{-2}
	Tn5B1-4	ND	ND	4×10^{-3}

*NT: non tested; ND: non detected

Table 5. Real-time qPCR quantification of *photolyase* and *gp64* genes in DNA extracted from occlusion bodies obtained in cell culture co-infected with AgMNPV and ChinNPV

Sample	**Viral DNA (ng/µl)**	
	ChinNPV – *photolyase*	**AgMNPV- *gp64***
Experiment 1		
P0	3.4×10^{-4}	3.46
P1	9.8×10^{-5}	3.89
P2	7.17×10^{-6}	1.97
Experiment 2		
P0	8.31×10^{-4}	3.25
P1	3.04×10^{-4}	4.06
P2	3.17×10^{-5}	1.79

Biological activity of OBs obtained from *in vitro* co-infection

Low mortality was observed in *A. gemmatalis* (6%) and *C. includens* (4%) larvae in the bioassay B1. Ten *A. gemmatalis* and ten *C. includens* larvae displayed symptoms of polyhedrosis disease, but they remained alive until 7 d.p.i. The qPCR analysis from larvae of B1 (Tables 6 and 7) detected only ChinNPV in the dead larvae (one *A. gemmatalis* and three *C. includens*), however, at low concentrations. AgMNPV was not detected in the dead larvae, and both viruses were not detected in diseased larvae. The reasons for these negative results could be mortality caused by other agents or that the quantity of viruses was below the limit of detection of the method used. It is relevant to consider that these samples had low total DNA concentration (1 to 5 ng/µl). In the bioassay B2, no dead or diseased *C. includens* larvae was observed until 7 d.p.i. Therefore, a qPCR of alive larvae was performed. Interestingly, both viruses occurred in the symptomless larvae, but at low concentrations, indicating survival with non-lethal dose (data not shown).

Table 6. Real-time qPCR quantification of *photolyase* and *gp64* genes in DNA extracted from dead *A. gemmatalis* larvae to confirm the co-infection *

Sample number	Viral DNA (ng/µl)	
	ChinNPV – *photolyase*	**AgMNPV- *gp64***
B1-1	1×10^{-5}	ND
B1-2	ND	ND
B1-3	ND	ND
B1-4	ND	ND
B1-5	ND	ND
B1-6	ND	ND
B3-1	7.31×10^{-6}	12.77
B3-2	4.91×10^{-6}	10.25
B3-3	5.19×10^{-6}	12.93
B3-4	8.21×10^{-6}	12.2
B3-5	1.36×10^{-5}	4.15
B3-6	2.84×10^{-5}	9.77
B3-7	1.14×10^{-4}	34.82
B3-8	6.1×10^{-4}	28.81
B3-9	1.5×10^{-3}	17.11
B3-10	5.16×10^{-4}	33.3
B3-11	3.12×10^{-4}	15.2
B3-12	1.07×10^{-4}	31.8
B3-13	1.1×10^{-4}	28.37
B3-14	4.41×10^{-4}	26.62
B3-15	2.14×10^{-4}	22.33

*Bioassay B1 performed with 100 larvae - Concentration of 10^2 OBs/ml
Bioassay B3 performed with 40 larvae - Concentration of 10^7 OBs/ml. Only larvae with mixed infection is shown.
ND – Non detected

In the bioassay B3, high mortality of *A. gemmatalis* larvae (91.5%) and low mortality of *C. includens* larvae (5.5%) was observed at 7 d.p.i. However, 13 (36%) *C. includens* larvae showed polyhedrosis disease symptoms and were collected for DNA extraction and qPCR. The qPCR results indicated that all dead *A. gemmatalis* larvae had high quantities of AgMNPV. On the other hand, only 15 of these dead *A. gemmatalis* larvae presented ChinNPV (Table 6), but in low quantities. This result reflects the differences observed in the quantities of each virus species in the inoculum (OBs collected from the P0 passage) (Table 5). The two dead *C. includens* larvae presented high amounts of ChinNPV and low quantity of AgMNPV (Table 7). However, note that all the live symptomatic *C. includens* larvae presented both viruses in similar quantity, except for one that displayed a high quantity of ChinNPV. In some of these larvae, the amount of AgMNPV was higher than the quantity of ChinNPV.

Table 7. Real-time qPCR quantification of *photolyase* and *gp64* genes in DNA extracted from dead or symptomatic *C. includens* larvae to confirm the co-infection *

Sample number	**Viral DNA (ng/µl)**	
	ChinNPV – *photolyase*	**AgMNPV- *gp64***
(dead larvae)		
B1-1	9.1×10^{-6}	ND
B1-2	3.7×10^{-5}	ND
B1-3	2.7×10^{-5}	ND
B1-4	ND	ND
B3-14	23	2.72×10^{-3}
B3-15	89.7	3.04×10^{-3}
(symptomatic larvae)		
B3-1	3.35×10^{-3}	2.47×10^{-4}
B3-2	4.3×10^{-4}	3×10^{-4}
B3-3	1×10^{-3}	3.8×10^{-4}
B3-4	5.47	1×10^{-4}
B3-5	6.35×10^{-4}	1.41×10^{-4}
B3-6	5.6×10^{-4}	7.73×10^{-4}
B3-7	1.5×10^{-4}	1.65×10^{-4}
B3-8	6.14×10^{-4}	6.7×10^{-4}
B3-9	1.5×10^{-4}	1.06×10^{-3}
B3-10	1.31×10^{-5}	1.53×10^{-4}
B3-11	5.46×10^{-4}	2.35×10^{-4}
B3-12	2.06×10^{-4}	6.44×10^{-5}
B3-13	9.75×10^{-4}	2×10^{-4}

* Bioassay B1 performed with 100 larvae - Concentration of 10^2 OBs/ml
Bioassay B3 performed with 40 larvae - Concentration of 10^7 OBs/ml
ND – Non detected

DISCUSSION

The ChinNPV isolate collected in the Brazilian Cerrado region (Buritis, MG) was first analyzed in terms of its restriction DNA profile and virulence. For biocontrol, a more important aspect than the genetic variation of the virus isolates is their phenotypic implications on pest management. The isolate ChinNPV-Buritis presented genetic heterogeneity revealed by the presence of submolar bands in the restriction endonuclease profile and a distinct *Eco*RI digestion profile, sharing characteristics with IE, IF, and IG isolates. Interestingly, the *in silico* analysis for IF and IG isolates revealed a different *Eco*RI profile compared to the description of Alexandre et al.[20]. The method applied for genome sequencing of these isolates[21] may have selected virus genotypes. Genetic heterogeneity of wild-type isolates is well documented for baculoviruses[20,31,32].

Although the pathogenicity is important to select an isolate, it does not always reflects its potential for biocontrol in the field, where the conditions are different and much more variable[33]. Other phenotypic features like OB production could be higher in less virulent isolates, as demonstrated for SfMNPV[34]. In the case of ChinNPV-Buritis, natural epizootics resulted in the control of 30% *C. includens* population in a soybean field[5].

The potential for *in vitro* production of this isolate was tested, because the difficulties of *in vivo* production of ChinNPV need to be overcome. Although this isolate was able to infect SF21, Sf9, and Tn-5B1-4 cell lines, the best OBs production was obtained in the last one. However, serial passages in *Trichoplusia ni* cells (Tn-5B1-4) were not successful and the infection appeared to be restricted to individual cells. According to Reid et al.[6], one of the requirements for a cell line to be considered for *in vitro* production of a baculovirus is its capacity to produce a useful virus at a yield of at least 300 OBs/cell. Therefore, improvements are necessary for further development of *in vitro* ChinNPV production.

Both AgMNPV and ChinNPV were able to infect cells originated from other insects (*S. frugiperda* and *T. ni*), although AgMNPV cannot cause a patent infection in *C. includens* larvae. Similarly, ChinNPV caused only covert infection in *A. gemmatalis* larvae. For a productive infection, cellular structures and molecular pathways must be compatible with the virus for all the major events in virus replication including attachment, entry, uncoating, replication, assembly, and exit. Insects have evolved methods to inhibit or block virus infections. These defense mechanisms include physical barriers along with local and systemic responses. The latter includes the production of antimicrobial peptides and highly specialized cells called hemocytes[35]. Resistance to AgMNPV was reported in laboratory selected populations of *A. gemmatalis* from Brazil and USA continuously exposed to the virus for 4 generations[36].

The *in vitro* and *in vivo* co-infection has been successfully achieved using different genotypes of certain baculovirus species[10,11,12]. The biological features of mixed virus populations includes increased pathogenicity[11] or increased pathogenicity and virulence[12], although some works described reduced pathogenicity due to the presence of defective genotypes[37].

Particularly in our co-infection study, a disproportionate amount of AgMNPV and ChinNPV was observed in the OBs used as inoculum. As a result, the amount of each virus varied considerably in the progeny. Maintenance of a certain genotype in the occluded virus

population as the progeny virions are passed in subsequent host generations depends upon individual larval cells becoming co-infected with both viruses. The infection in an individual larva can be initiated with a disproportionate mixture, producing high variation in the percentage of virus genotypes[10]. The challenge presented for co-infection of AgMNPV and ChinNPV is to find a common and suitable host, either *in vitro* or *in vivo*.

The qPCR developed in this study proved to be a useful tool to detect and quantify these two viruses, AgMNPV and ChinNPV in several conditions: from purified OBs, infected larvae, infected cells, or from BVs in the cell culture supernatant. We also efficiently detected the viruses from larvae hemolymph (data not shown). The qPCR is a faster and more sensible method than the traditional techniques to quantify viral genotypes in BVs and OBs, although it is more expensive. In addition, AgMNPV is used for biological control in Brazil as biopesticides formulations, which present difficulties to traditional OBs quantification. We successfully detected AgMNPV from a formulated wettable powder with the qPCR technique (data not shown). The qPCR method is useful for a wide range of applications in field and laboratory. Barrera et al.[29] developed a qPCR that detected granulovirus from samples of larva and soil, plus determined the virus concentration in the pesticide formulated as emulsifiable concentrate. According to the authors, this technique is reproducible, sensitive, and specific to allow viral persistence studies in the field, viral infection control in insect rearing, and quality control of a biopesticide based on baculovirus. In addition, qPCR is a powerful technique to detect viruses in asymptomatic insects. The persistence of viruses in natural populations, as the result of surviving a non-lethal virus challenge may play an important role in epizootics[33] and might be better investigated with this technique.

CONCLUSION

ChinNPV-Buritis is a potential isolate for the development of biopesticide. *In vivo* and *in vitro* production studies with this isolate are being conducted for further formulation and field application. The methods presented here can be applied in future co-infection assays and open new possibilities of future research to investigate the effect of non-lethal doses and the implications on vertical transmission of ChinNPV and AgMNPV.

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