Isolation and Characterization of a Nucleopolyhedrovirus from *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae)

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Abstract *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae) is an important pest of vegetable crops widely distributed in South America. At present, its control is based on the use of broad spectrum chemical insecticides. In order to develop a more selective alternative to be applied in integrated or organic pest management programs, we isolated and characterized a baculovirus that infects *R. nu*, denominated RanuMNPV (*Rachiplusia nu* multiple nucleopolyhedrovirus) in view of its morphology and pattern of infection. According to optical microscope images of *in vitro* infected cell-cultures, data from bioassays carried out with various species of larvae, and DNA sequences from *p74*, *polyhedrin*, *v-cathepsin*, *v-chitinase*, *lef8* and *lef9* genes, this isolate could be considered as a variant of *Autographa californica* MNPV with a different host range.

Keywords RanuMNPV, AcMNPV, Biological Control, Host Range

1. Introduction

The family Baculoviridae comprises a large number of viruses which infect arthropods, mostly insects from the order Lepidoptera and some others from Hymenoptera and Diptera[1],[2]. The viral genome is double stranded circular DNA, with sizes ranging from 80 to 180 kbp. Mature virions, assembled late in the infectious cycle, are surrounded by a crystalline proteinaceous matrix. These structures are known as occlusion bodies (OB), and their morphology and size have been used to subdivide the Baculoviridae into two genera: nucleopolyhedrovirus (NPV) and Granulovirus (GV). More recently, on the basis of genome analysis and phylogenetic studies, a new classification and nomenclature was proposed and accepted. The updated classification includes four genera: *Alphabaculovirus* (lepidopteran-specific NPV), *Betabaculovirus* (lepidopteran-specific GV), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV)[3]. Viruses grouped within the genus *Alphabaculovirus* produce OBs constituted by polyhedrin as the major matrix protein and several enveloped virions containing a single (SNPV) or multiple (MNPV) nucleocapsids. *Alphabaculovirus* is further divided into Group I or Group II, considering sequence analyses and the presence of *gp64* gene[4].

Among the NPVs isolated from noctuid larvae of the Subfamily Plusiinae, many have demonstrated a great potential as biopesticides and are successfully used in pest management programs[5-11]. Studies based on restriction endonuclease mapping, nucleic acid hybridization, and complete or partial sequencing of MNPVs isolated from *Autographa californica* (AcMNPV), *Rachiplusia ou* (RoMNPV) and *Anagrapha falcifera* (AfMNPV), revealed the existence of a close genomic relationship among them[12-15]. For that reason, RoMNPV and AfMNPV were proposed as variants of AcMNPV[1], although they present differences in host range and virulence[16].

The looper *Rachiplusia nu* (Guenée, 1852) (Lepidoptera: Noctuidae, Plusiinae), is a polyphagous leaf-feeder species widely distributed in South America. Larvae cause damage in several economically important species, such as sunflower, tobacco, soybean and vegetable crops. At present, the management of *R. nu* is based on the applications of broad spectrum chemical insecticides. For many years, studies have been focused on the finding of alternative biological control methods. In this regard, isolates of NPVs from *R. nu* were evaluated under laboratory and field conditions[17-19]. However, none of them were fully characterized.

With the objective of proposing a bio-pesticide for the control strategy of *R. nu,* we isolated and characterized a *Rachiplusia nu* nucleopolyhedrovirus in terms of its major biological, morphological, and biochemical properties. The relationship among this isolate (named RanuMNPV),

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AcMNPV and other genomic variants of the baculovirus prototype are also discussed.

2. Materials and Methods

2.1. Larval rearing and Baculovirus Production

Larvae of *Rachiplusia nu* were obtained from a colony established in our laboratory (IMyZA-INTA), and maintained on artificial diet at 25 ± 1℃, 14:10 h light-darkness (LD) cycle, and 60% relative humidity (RH)[20]. The virus described in this study was isolated from diseased *Rachiplusia nu* larvae field collected in Oliveros (Santa Fe Province, Argentina). The virus was propagated in the host by infecting early fourth-instar larvae by diet surface contamination (1,500 OBs/mm^2). OBs were purified from aqueous homogenates of dead larvae by two rounds of centrifugation in continuous 40-65% (wt/wt) sucrose gradients, at 100,000 g for 1 h at 4℃. The OB-containing fraction was collected, diluted with distilled water, and concentrated by centrifugation at 8,000 g for 20 min, at 4℃. The pellets were resuspended in sterilized double-distilled water and conserved at -20℃. The quantifications of OBs were performed by calculating the mean of five independent counts using hemocytometer (Neubauer chamber) and optic microscopy (Nikon Eclipse TS 1000).

2.2. Morphological Studies

Purified OBs from infected *R. nu* were embedded in 2% (wt/v) agarose and fixed in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) - 0.25 M sucrose for 3 h. Post-fixation was carried out for 2 h in 1% osmium tetroxide in 0.1 M cacodylate buffer. Then, samples were embedded in Epon-Araldite after being dehydrated through an ethanol-propylene oxide series. For phase contrast light microscopy, sections were cut 1 μm thick and examined unstained. For transmission electron microscopy, sections about 70 nm thick were stained in 5% (v/v) aqueous uranyl acetate for 30 min, lead citrate for 10 min, and examined with a JEM-1200 EX II electron microscope.

2.3. Recovery of Budded Virions for *in Vitro* Assays

Purified OBs from infected *R. nu* were used for genomic DNA isolation. Briefly, virions were released in 0.1 M Na_2CO_3, 0.17 M NaCl, 0.01 M EDTA (pH 10.5) at 37℃, for 30 min. After this, the suspension was neutralized with 1 N HCl and undissolved polyhedra were removed by low–speed centrifugation. Then, 0.5% (wt/v) SDS and proteinase K (0.25 mg/ml) were added, incubating at 37℃ for 3 h. Finally, DNA was isolated by phenol extraction and concentrated by ethanol precipitation[21].

Spodoptera frugiperda (Sf9) cells[18, 37] were grown at 27℃ in GRACE's medium (Invitrogen) containing 10% fetal bovine serum (Bioser) and supplied with antibiotics and antimycotics (Invitrogen). To produce budded viruses (BV) of *R. nu* baculovirus, a total of 1 x 10^6 Sf9 cells were seeded in 60 mm plate and incubated at 27℃ for 1 h. Six microliters of CellFECTIN^{TM} (Gibco Life Technologies) were diluted in 100 μl of serum-free GRACE's medium, mixed with viral DNA (1-2 μg diluted in 100 μl of serum-free GRACE'S medium), incubated at room temperature for 40 minutes and diluted with 800 μl of serum-free GRACE's medium. After removing the culture medium, the DNA/CellFECTIN mix was added to the cell monolayers and incubated at 27℃ for 5 h without agitation. Subsequently, 2 ml of GRACE'S medium with 10% fetal bovine serum were added and the cells incubated at 27℃. Seven days post-transfection the conditioned media containing BVs were collected, centrifuged for cell debris clarification and stored at 4℃ until use. Two rounds of virus amplification were performed using semi-confluent Sf9 cell monolayers and recovery BVs[multiplicity of infection (moi): 0.1].

2.4. Viral Genome Size Determination

Sixty percent confluent monolayers of Sf9 cells growing in previously mentioned culture conditions were infected with *R. nu* baculovirus, (moi: 0.1) and incubated for 7 days at 27℃. Then, BVs were collected from conditioned media, centrifuged for cell debris clarification and resuspended in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.25% SDS). After a 37℃ overnight treatment with proteinase K (20 mg/ml), virus DNAs was purified by standard phenol/chloroform extraction and ethanol precipitation[21]. The DNA was quantified by spectrometry (OD260, SmartSpec 3000 BioRad) and was tested by electrophoresis in 0.6% (wt/v) agarose gel and ethidium bromide staining. The *R. nu* baculovirus genome size was estimated through restriction analysis using BamHI, EcoRI, HindIII, KpnI and XhoI (Invitrogen) and subsequent agarose gel electrophoresis using appropriate molecular weights as references (PB-L).

2.5. Cloning and Partial Sequencing of *Polyhedrin, p74, v-cathepsine, v-chitinase, lef8 and lef9 genes*

With the goal to classify *R. nu* baculovirus, some auxiliary and core genes were partially sequenced. Thus, aliquots of *R. nu* baculovirus genome were independently digested using HindIII and EcoRI restriction endonucleases (Invitrogen), pZErO 2.0 (Invitrogen) was selected as molecular cloning vector, digested with the same enzymes, and dephosphorylated using alkaline phosphatase (Invitrogen). Then, *R. nu* baculovirus restriction endonuclease fragments were cloned using T4 DNA ligase (Invitrogen) and *Escherichia coli* Top 10 (Invitrogen). Later, the HindIII and EcoRI genome libraries were screened to detect *polyhedrin* and *p74* genes by Dot blot assays (BioRad). To find the *polyhedrin locus* a heterologous probe deriving from *polyhedrin* orthologous gene of AgMNPV was used[25]. To find *p74*, the probe was based on a PCR product derived from of *R. nu* baculovirus *p74* gene obtained using Taq DNA polymerase (PB-L) and

universal primers[26]. Both fragments were purified using QIAEX II Gel extraction Kit (QIAGEN), labeled using alkaline phosphatase, and the hybridizations were performed according to the manufacturers´ specifications (AlkPhos direct, Genes Images from Amersham Pharmacia Biotech). Moreover, fragments of *v-cathepsin*, *v-chitinase*, *lef8* and *lef9* genes were amplified by PCR using Taq DNA polymerase and the primers p_cat398/p_cat896, p_quit68 / p_quit1513, prL8-1/prL8-2 and prL9-1/prL9-2, repectively; the reaction conditions used were the previously described[27],[28]. After this, amplicons were cloned in pGemT-easy vector (Promega). All molecular clones containing fragments of the six genes tested were recovered and the inserts sequenced using universal primers (Macrogen, http://www.macrogen.com).

2.6. Sequence Analyses

Sequence analyses were performed using available genome sequences of baculoviruses (www.ncbi.nlm.nih.gov). Multiple alignments were obtained with ClustalX program, using Gap opening 10 and Gap extension 0.1 as parameters for pairwise alignments, and Gap opening 10 and Gap extension 0.05 for multiple alignments. Dayhoff distance corrected UPGMA distance analyses (gamma shape parameter: 2.25; gaps/missing data: complete deletion, respectively) and phylogenetic trees (1000 bootstrap replicates) were inferred from the amino acid sequence alignments by using MEGA, version 4.0[29],[30]. The pairwise comparisons of amino acid sequences were performed using ClustalW with the default parameters. Distance matrices from aligned nucleotide sequences were determined by using the Pairwise Distance calculation of the same software applying the Kimura 2-parameter (K-2-P) model[30].

2.7. Larval Bioassays

Rachiplusia nu pre-molted second-instar larvae were placed into individual wells (1 cm³) of a 50 multiwell device containing artificial diet (without formalin) contaminated on surface with *R. nu* baculovirus´s OBs (2, 8, 32, or 128 OBs/mm²). Then, larvae were incubated at $25 \pm 1℃$, 14:10 LD and 50% RH. Mortality was recorded daily until pupation. Three replicate assays of 50 individuals each one were carried out for the different tested doses. Other 50 additional individuals were used as controls. The concentration-mortality response data were analyzed by *probit*[22] using MSTAT-C, and indicated as LC_{50} (Lethal Concentration 50) values. For comparative purposes, equivalent bioassays were performed against two major regional soybean pests: *Anticarsia gemmatalis* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Thus, fifty third-instar larvae were individually placed into 40 ml plastic cups that contained surface contaminated diet (3,000 OBs/mm²); while 30 individuals were used as controls. In any case, mortality by baculovirus infection was verified by optic microscopy examination.

2.8. *In Vitro* Cell Culture Host Range

BVs of *R. nu* baculovirus and AcMNPV were amplified and tittered by plaque assay in Sf9 cells[23]. The virus infectivity in Sf9, Sf21 (both derived from *Spodoptera frugiperda*), Hi-5 (derived from *Trichoplusia ni*) and UFL-Ag-286 (derived from *Anticarsia gemmatalis*)[24] cell lines were tested. To accomplish this, cells growing under previously mentioned conditions at 60% confluence in 24 multiwell plates (Sarstedt) were exposed with *R. nu* baculovirus or AcMNPV (moi: 0.5). After incubating during 4 days, cells were photographed using an optic microscope (Nikon Eclipse TE2000) and the conditioned media harvested in order to quantify BV productions by a colorimetric assay. Briefly, Sf9 cells growing in 96 multiwell plates were treated with the virus samples and 4 days incubated. Then, cells were fixed using 10% (v/v) methanol for 10 minutes, and stained by 0.1% (wt/v) violet crystal treatment. Finally, absorbance measures at 570 nm (Dynex Technologies MRX tc Microplate Reader) were performed and relativized respect to previously plaque assay tittered AcMNPV and *R. nu* baculovirus stocks.

3. Results and Discussion

3.1. Morphological Studies of RanuMNPV

R. nu infected larvae with a field isolated virus were studied to analyse some morphological properties. Thus, electron microscope examination of ultrathin sections revealed the presence of OBs with typical polyhedron morphology that averaged 1.52 μm (+/- 0.3) in diameter. Each occlusion body contained several virions with one to eight nucleocapsids (Figure 1). These data are in agreement with the virus description for multiple nucleopolyhedroviruses (MNPVs) of Lepidoptera[2],[3]. Consequently, the *Rachiplusia nu* virus isolate was denominated RanuMNPV (*Rachiplusia nu* multiple nucleopolyhedrovirus).
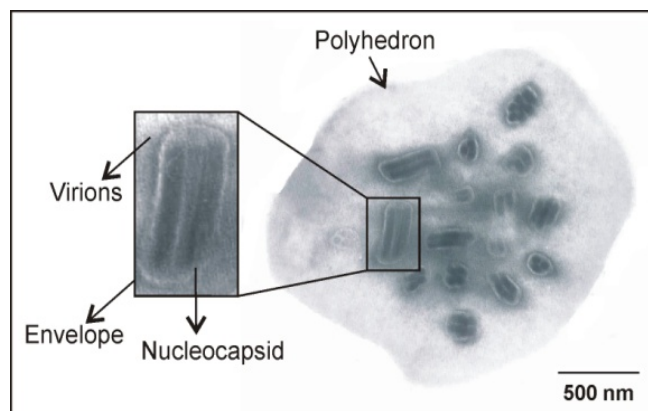
Figure 1. Electron micrograph of an occlusion body of RanuMNPV, showing longitudinal and cross sections of virions containing multiple nucleocapsids. Bar: 500 nm

3.2. RanuMNPV Genome Analysis and Classification
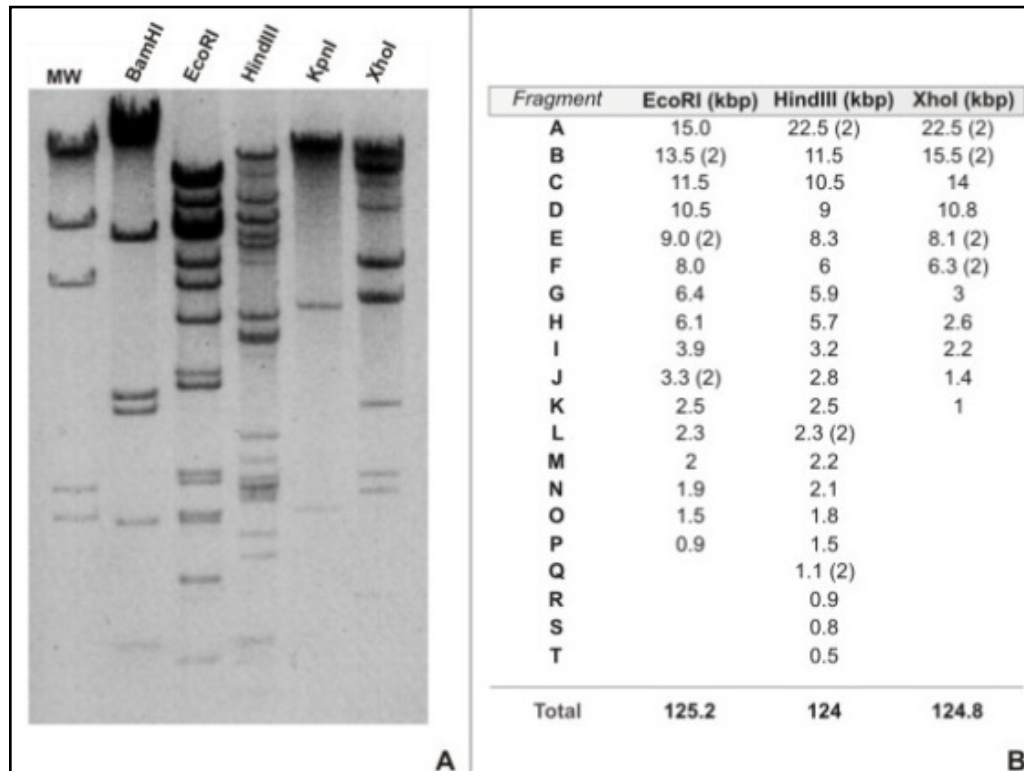
Figure 2. **A.** Restriction fragments of RanuMNPV DNA genome resolved by electrophoresis in 1 % agarose. The enzymes used are indicated on the top of each lane. MW: Molecular Weight (λ DNA digested with HindIII). **B.** Size estimation for RanuMNPV DNA fragments and whole genome (R^2: 0.962). The table shows the assigned letter to each different restriction fragment (A to T) from EcoRI, HindIII and XhoI endonucleases and the estimated size in kbp. Total size was calculated from the sum of all different fragments. BamHI and KpnI digestions were not considered due to the low abundance of target sites in the genome

| Fragment	EcoRI (kbp)	HindIII (kbp)	XhoI (kbp)
A	15.0	22.5 (2)	22.5 (2)
B	13.5 (2)	11.5	15.5 (2)
C	11.5	10.5	14
D	10.5	9	10.8
E	9.0 (2)	8.3	8.1 (2)
F	8.0	6	6.3 (2)
G	6.4	5.9	3
H	6.1	5.7	2.6
I	3.9	3.2	2.2
J	3.3 (2)	2.8	1.4
K	2.5	2.5	1
L	2.3	2.3 (2)	
M	2	2.2	
N	1.9	2.1	
O	1.5	1.8	
P	0.9	1.5	
Q		1.1 (2)	
R		0.9	
S		0.8	
T		0.5	
Total	**125.2**	**124**	**124.8**

RanuMNPV genome was isolated from BVs produced by *in vitro* cell culture infections and later fragmented by endonuclease restriction enzyme digestions with BamHI, EcoRI, HindIII, KpnI or XhoI in order to estimate the DNA size, which was 124,7 (+/-0,6) kbp (Figure 2). Simultaneously, two genome libraries of RanuMNPV DNA were generated with the aim to find and sequence two important late genes of NPVs: *polyhedrin* and *p74*. The first sequence encodes the most important polypeptide present in the crystal proteinaceous core of *Alphabaculovirus* OBs; while *p74* or *pif-0* encodes the most studied *per os* infectivity factor of Baculoviridae and its evolution could be associated with insect hosts[31]. According to this, a RanuMNPV library was screened using a heterologous *polyhedrin* probe (orthologous *locus* from AgMNPV). Several clones were detected and sequenced (GenBank DQ345451). The subsequent analysis showed the presence of a 738 bp open reading frame (ORF) that theoretically encodes a polypeptide composed by 245 amino acids length with a molecular weight of 28,641.3 Da. The nucleotide sequence comparison respect to the AcMNPV ortholog revealed 100% of identity.

Otherwise, to obtain the sequence of RanuMNPV *p74* gene the genome libraries were screened using a homologous probe (PCR fragment containing a partial sequence of RanuMNPV´s *p74* gene). Several clones were detected and sequenced (GenBank DQ345452). Thus, this *locus* revealed the presence of a 1,938 bp ORF that theoretically encodes a protein of 645 amino acids length, with a molecular weight of 73,980 Da. Comparison of RanuMNPV vs AcMNPV *p74* nucleotide sequences showed only four differences (A_{218} to T_{218}, C_{475} to T_{475}, C_{1776} to A_{1776} and A_{1777} to C_{1777}). The change at position 1776 (amino acid 592) is silent; but the other three nucleotide differences derive in three amino acid changes: E_{73} by V_{73}, C_{95} by T_{95} and M_{593} by L_{593}.

On the other hand, fragments of two auxiliary genes (*v-cathepsin* and *v-chitinase*) were amplified by PCR and sequenced, showing 100% identity respect to the corresponding AcMNPV orthologs.

In view of previous results, all suggesting that RanuMNPV would be a variant of AcMNPV, the accepted Baculoviridae classification method and the kimura 2 parameters model were applied[28],[30]. So that, PCR fragments amplified from RanuMNPV genome containing partial sequences of *lef8* and *lef9* genes were obtained. These products were molecular cloned and subsequently sequenced, newly detecting 100% identity respect to AcMNPV orthologs. In consequence, RanuMNPV and AcMNPV should be considered variants of the same viral species. Anyway, the phylogenetic relationships among *Alphabaculovirus* Group I members were inferred based on an amino acid concatemer P74-Polh-Cath-Chit-Lef8-Lef9) (Figure 3).
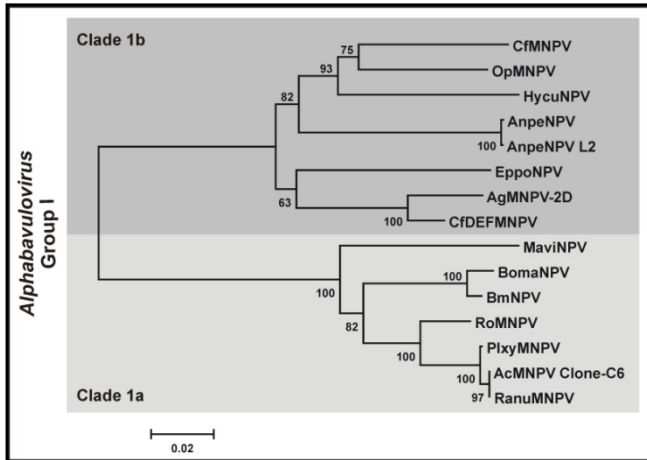
Figure 3. Phylogeny of Group I *Alphabaculovirus*. Sequences of P74, Polyhedrin, Cathepsine, Chitinase, Lef8 and Lef9 proteins from RanuMNPV and the corresponding orthologous sequences from other 14 Group I alphabaculoviruses were concatemerized and a phylogeny was inferred using the Mega 4.0 software. The accepted clades (1a, 1b) are indicated and highlighted by different shades of gray

Because of its very close proximity with AcMNPV, RanuMNPV grouped with the clade 1a. It is important to note that these viruses present high nucleotide identities among them (97-99.9%) and a broad host range[32]. In fact, as an example of their versatility in the ability to infect new hosts, only two amino acid changes into the *DNA helicase* gene were required for AcMNPV infecting *Bombyx mori* larvae[33].

3.3. Host Range Studies

Considering previous data and with the aim to characterize the RanuMNPV host range, larval bioassays in various lepidoteran were carried out. Thus, the LC_{50} of RanuMNPV in *Rachiplusia nu* larvae was 10.4 (range: 8.5 to 16.2) OBs/mm^2. In contrast, *Spodoptera frugiperda* larvae were not susceptible to RanuMNPV under the tested conditions, while 100% mortality was obtained against *Anticarsia gemmatalis* larvae considering percentage of mortality and disease development as criteria of susceptibility. In view of that, new bioassays using doses from 8 to 2,048 (rate: 4) OBs/mm^2 were carried out against *Anticarsia gemmatalis*, allowing to estimate a value of 410 OBs/mm^2 as LC_{50}. This result was different to previous reports, suggesting that RanuMNPV had a differential host range respect to AcMNPV[34].
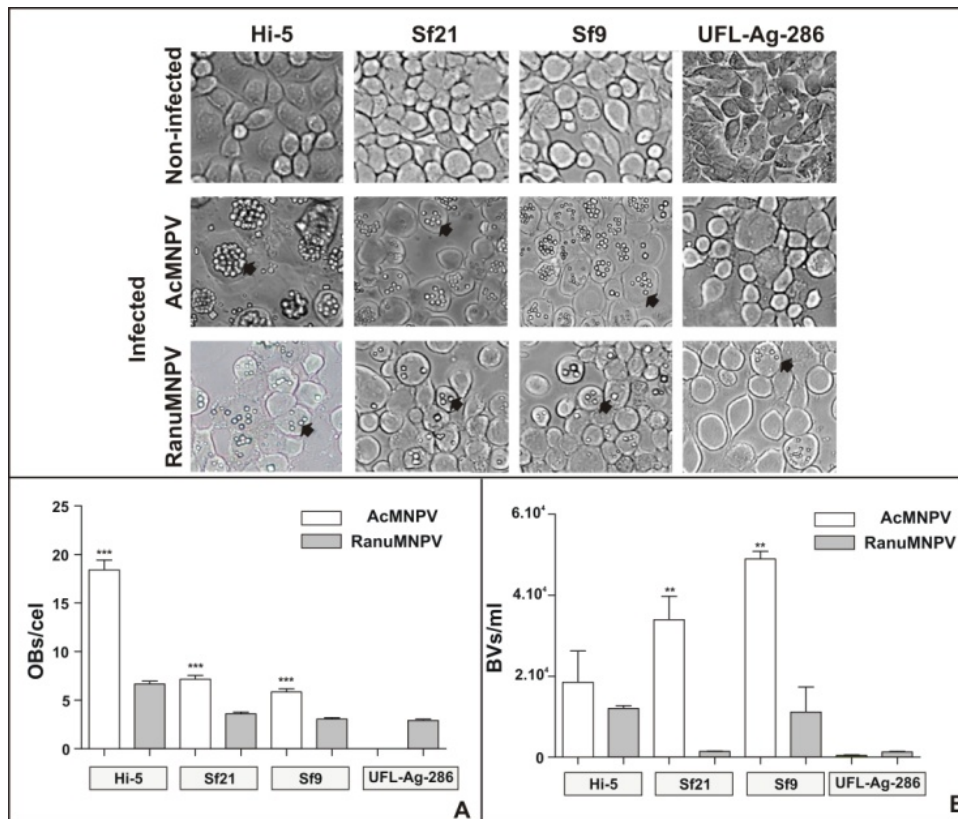
Figure 4. **A.** Insect cells (Hi-5, Sf21, Sf9, UFL-Ag-286) growing in GRACE´s medium were not inoculated or inoculated with AcMNPV or RanuMNPV budded viruses (moi 0.5) and incubated at 27ºC for 4 days. Cells were photographed to detect presence of occlusion bodies (OBs). All pictures correspond to 300X magnification. OBs are indicated with black arrows. **B.** The bar graphic shows the average number of OBs/cell, corresponding to 20 infected cells for each assay. The error bars are indicated and represent the standard deviation (***p<0.0001, Student's *T test*). **C.** Average number of BVs/ml corresponds to 6 replicates. The error bars are indicated and represent the standard deviation (**p<0.005, Student's *T test*)

Moreover, to verify the infectivity of RanuMNPV BVs in *in vitro* cell cultures various infection assays in Sf9, Sf21 (both from *Spodoptera frugiperda*), Hi-5 (from *Trichoplusia ni*) and UFL-Ag-286 (from *Anticarsia gemmatalis*) cells were performed. Four days post-inoculation, cells were analyzed by light microscopy (Figure 4A). All tested cell lines exposed to RanuMNPV showed a typical cytopathic effect, even UFL-Ag-286; this was not observed when AcMNPV was used, which clearly showed presence of OBs in Sf9, Sf21 and Hi-5 cells but not into the *A. gemmatalis* derived cell line. Specially, the numbers of OBs per infected cell produced by AcMNPV and RanuMNPV were significantly different for all tested cell lines, suggesting that the new isolate had a particular pattern of infection and productivity (p<0.0001) (Figure 4.B. and C.). In all tested cases, higher BV productivities were observed when cells were infected with AcMNPV, only with the exception of RanuMNPV in UFL-Ag-286 cells. Probably, the detected differences in the capabilities of these NPVs to establish productive infections in tested cell lines may be due to the presence or absence of cellular or viral factors necessary for genome replication, assembly and/or virus budding.

Despite the extremely high sequence similarity found between RanuMNPV and AcMNPV, the experimental host ranges studied in laboratory conditions revealed to be significantly different suggesting that other non detected sequence variations occur.

4. Conclusions

The isolation and partial characterization of a baculovirus obtained from a single diseased *Rachiplusia nu* larva is described by this work. The structural and molecular studies showed the presence of Multiple Nucleopolyhedrovirus (MNPVs) into the cell nuclei of affected tissues that contained nucleocapsids carrying genomes with a size estimated in $\cong$ 125,000 bp. According to the accepted strategy to assign species and genus for new baculovirus isolates[27],[28], total (*p74* and *polyhedrin* genes) and partial (*v-cathepsine*, *v-chitinase*, *lef8* and *lef9* genes) sequences were obtained and analyzed. Thus, the Kimura 2 parameter model was applied showing that RanuMNPV would be considered as a new variant of AcMNPV species, belonging to the Clade 1a of Group I *Alphabaculovirus* genus. Despite this classification, the performed biological assays revealed a different behavior between AcMNPV and RanuMNPV, consequently exposing a dissimilar host range. Probably, the standard baculovirus species assessment should be reconsidered in future, involving the comparisons of more conserved sequences and including some phenotypic factors such as structural dimensions and host range. Although more evidences are needed, considering our results it is possible to speculate that the differences in *p74* gene be important in the larva infectivity. That protein is an important *per os* infectivity factor and possibly few

amino acid changes may be useful and necessary to support primary infections in new hosts. However, other variations in RanuMNPV genome should be occur because BV forms supported the infection into *A. gammatalis* derived cell lines, multiplication cycle that not require of viral factors crucial for the late baculovirus phenotype. For these reasons, the complete genome sequence of RanuMNPV appears as the next step to complete its characterization, and thus understand what genotypic differences may be crucial in host infectivity.

This report shows the versatility and variability of AcMNPV and its variants in nature, and provides information that can be applied to optimize the use of baculoviruses as biological control agents of agriculture pests. In particular, RanuMNPV is a new baculovirus isolate that may be proposed as an alternative to control *Rachiplusia nu* larvae. Besides, this virus adds another useful candidate which together with AgMNPV can attend in controlling *Anticarsia gemmatalis*, one of the most important pests in South America.

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