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GENOMIC HOMOLOGY AND MIXED INTERACTIONS OF BACULOVIRUSES INFECTING THE EGYPTIAN COTTON LEAF WORM *SPODOPTERA LITTORALIS* *IN VIVO* AND *IN VITRO.*

[1]

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Abstract

Baculovirus genomes from different virus species generally exhibit a considerable degree of structural diversity. However, some sequenced baculovirus genomes from closely related viruses are structurally very similar and share over-all nucleotide sequence identities in excess of 95%. This work focuses on the comparative essential basis studies to better know complex interactions between baculoviruses infecting the pest in question (*Spodoptera littoralis*) in relation to stability or mechanism of infection in the same host multiple nucleopolyhedroviruses (MNPVs) Spli MNPV, and granulovirus SpliGV. Mixed infection of the tow viruses was carried out *in vivo* and *in vitro*, showing clearly that the mixture with Spli GV enhance the effect of Spli NPV when it was introduced 24hr prior to NPV or simultaneously. In addition to protein profiles by SDS-PAGE, TEM electron microscopy ultrathin sections and hybridization dot blots, gel transfer to follow the probable mechanism especially with the high percentage of genome homology that was over 66% with one of five tested restriction enzymes. These observations suggest an important possible role for recombination in the early evolution and biological characteristics of these two viruses.

Key words: Baculovirus, *Spodoptera littoralis*, SDS-PAGE, TEM , ultrathin sections and hybridization dot blots

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INTRODUCTION

Baculoviruses are promising viral insecticides and are safe for the environment. Interaction of baculoviruses *in vitro* and *in vivo* is a basic molecular and ecological question that has practical applications in agriculture(Cheng and Lynn 2009),Cellular secretion is also a fundamental property in cell-cell communication, recent investigations on how baculoviruses interact with insect cells and insect hosts focused particularly on a new interaction mechanism in which a secretion from cells infected with one virus enhance infection by a second virus. Regarding that Baculoviruses are important natural enemies of many lepidopteran and hymenopteran species and several have been developed as biological control agents of a range of pests in agriculture and forestry (Kempa *et al* 2011). They are known to be able to persist in the environment for long periods of time as occlusion bodies (OBs)Abdalla *et al* 2000),

if protected from ultra violet irradiation, allowing transmission within and between generations (Cory and Myers, 2003). In addition to this environmental reservoir, baculoviruses have been shown to persist in insect populations as covert or persistent infections which can be passed vertically from parent to offspring (Hughes *et al.*, 1997, Burden *et al.*, 2002, 2003 and Vilaplana *et al.*, 2010), although the mechanism by which the virus remains as a persistent infection and its role in virus population dynamics are unknown.

It was also suggested the possibility that covert infections might be more common in viruses that are only partially infective for a particular insect species, and that covert infection could result from exposure to baculoviruses from other species, that cannot infect on their own, but might under specific circumstances be introduced by a fortuitous co-infection event. There are other data that show that baculoviruses that under normal circumstances cannot infect alone, can be maintained in virus populations by co-occlusion and where co-infection can increase (or decrease) resulting host mortality (López-Ferber *et al.*, 2003). However, these interactions are usually among strains of one species of baculovirus, some of which lack key infection genes, rather than two different species, neither of which have been shown to lack known virulence genes (De Jong *et al.*, 2005 and Lauzon *et al.*, 2005).

Rohrman (2011) discussed the mechanism of infection and stated that there are two distinct physical types of infectious baculovirus particles: occlusion-derived virus (ODV) and budded virus (BV). Both ODV and BV contain rod shaped nucleocapsids that are assembled within the nucleus. ODV nucleocapsids are enveloped within the nucleus and occluded within a matrix of viral protein (polyhedrin or granulin) to form occlusion bodies (OB) or polyhedra/ granules. BV nucleocapsids exit the nucleus and acquire an envelope derived from the plasma membrane of the host cell upon budding through the membrane. ODV virions infect the host insect's midgut epithelial cells when OBs are ingested by the host and solubilized in the midgut lumen, releasing the ODV. BV that is assembled during the primary infection of midgut cells (and also during subsequent secondary infection of other tissues) serves as a vehicle to spread infection to other susceptible tissues in the host **(Harrison *et al.*, 2010)**. ODV are highly infectious to midgut epithelial cells and initiate the infection cycle in the animal. In contrast to the ODV, virions of the BV phenotype are generated earlier in the infection cycle, when nucleocapsids bud through the host cell plasma membrane into the hemocoel. In the hemocoel, BV initially infects the tracheal epithelium and hemocytes and then infects the fat body, muscle, malpighian tubules, and other tissues, leading to the eventual death of the host **(Lung *et al.*, 2003)**.

So, regarding all the precedent proposed analysis of complex mechanism of baculovirus our work is discussing and tries to understand the mechanism of the obscuring interaction, the influence and/ or impact of mixed infection by SpliGV and SpliNPV on each other and on their own host *Spodoptera littoralis* when existing together *in vivo* and *in vitro* at the level of the cells and the efficacy taking in consideration the possibility of resembles or homology between them as members of the same viral family.

MATERIALS AND METHODS

Mixed infection experimentations were carried out in different treatments, and all the treatments were realized using the LC25 of each virus. Treatments were as followos: The mixed infection *in vivo* between two isolates (NPV-NPV) which was performed on second instar (L2) and fourth instar (L4) pre-molting larvae of *S.littoralis* laboratory adapted strain reared on semi-synthetic medium originated from Fayiom field governorate. The dual infection concerning the mixed-infection

NPV-GV *in vivo* and *in vitro* SlNPV-SlGV were tested in two biological experimentations by time intervals treatments: the 1st was simultaneous infection and the 2nd is successive:

a) GV and NPV were applied at the same time (simultaneous).
b) NPV was applied 24hrs prior to GV.
c) GV was applied 24hrs prior to NPV.

Treatments were observed daily and percentages of mortality were registered, dead larvae and moribund were selected depending on the symptoms and separated in categories for the purification. DNA extraction and analysis was undergone on the resulted viral yields from infections which were purified prior to the DNA extraction to separate the two viruses. Samples of infected larvae and cells were subjected to viral purification and DNA extraction for the identification of the resulted virus from infection (Lery *et al* 1997, Abol-Ela *et al* 1998, Khamiss *et al* 1999, Lery *et al* 1999, and Khamiss 2005).

As for the viral infection *in vitro,* five cell lines were treated from Lepidopterous cell lines SlOmi Sl52, Sl95, Sl96, Sf9, Ld, these types of cells were obtained from the center of virology Faculty of Agric. Cairo Univ. Cell lines treatments were carried out by virions inoculation in C35 Petri dishes 3.5cm in diameter $2x10^5$ cell/dish which contain 2ml Grace's modified medium. Cells were observed daily and counted five times within 15 days. Cell viability was detected using viability staining (trypan blue). The cytopathic effect of cell (polyhedra in the nucleus or any other cytopathic effect) was observed and photographed under a phase-contrast inverted microscope (lieca) at 450X.

Samples *in vivo* and *in vitro* which were viral infected and had symptoms with time intervals (of 12hr, 24 hr, 72 hr, 5days and 15 days post infection (PI)) were prepared for the observation by the transmission electron-microscope and ultrathin sections to follow the mechanism and development of infection.

Electron microscope preparations:

Petri dishes of 35 mm which were seeded with $2x\ 10^5$ cells per 2 ml, incubated for 24 hr at 27° C and infected with Spli NPV, Spli GV, or Mixed at a multiplicity of infection (MOI) of 100 TCID. After 4-hrs adsorption period, the cultures were washed three times with 1 ml of Grace's medium then were incubated at the previously mentioned intervals, and the cells were fixed (**Knudson and Tinsley, 1974**). They were examined by phase contrast-light inverted microscope at first.

Cells were removed from the surface of the culture vessel by scraping with a rubber policeman and were pelleted at 1000 rpm for 10 min. Cells which were removed and pellet were fixed at 4° C in 5% (vol/vol) glutaraldehyde- sodium Cacodelate for 1 h followed by two washes and a second fixation in 2% (wt/vol) osmium tetroxide for 1hr. The fixed pellets were dehydrated through a graded series of ethanol and were embedded in agarose 2% (modifications of techniques were adapted in the lab. Sections were made (with a LKB ultratome) using diamond knives The sections were collected on 200-mesh copper grids and stained in 2% (wt/vol) uranyl acetate followed by lead citrate (REYNOLDS 1963, Washburn 2003). The specimens were examined in Zies electron microscope.

Total Spli NPV and SpliGV specific probes (and another from *Autogmpha califûrnica* NPV for the comparison in homology study) were prepared for the survey and differentiate the individual and mixed infection using the cold (Boehringer digoxigenin labeling system by Roche). Dot blot were carried out for the diagnosis and detection of viral infection (fig.4), and Southern hybridization from an agarose gel contain the SlGV digested by five restriction enzymes bands (HindIII, Bgl II, Pst I, EcoRI, and EcoRV) were transferred to 2

nylon membrane (Hybond) that are lit with genomic probes of SpliNPV and AcNPV which were previously mentioned and prepared (fig.6) to show the homology between the two viruses SpliNPV and SpliGV. The percentage of genomic homology was calculated depending on the molecular weight of positively reacted fragments correlated to the total restriction fragments molecular weight represented in each enzymatic profile.

RESULTS

The results of *in vivo* infection demonstrated in fig.(1) and table (1) indicated that mixing of two viruses gave percentage of mortality 3.4 times more effective than treatments individually by each virus. Regarding the timing of mixing the infection, the histogram showing clearly that the mixture with Spli GV enhance the effect of Spli NPV when it introduce 24hr before the NPV or simultaneously.

Table 1: Demonstrate the number of different categories of treatments by SpliNPV, Spli GV and mixed infection.

Treatment[a]	Symptom of NPV	Symptom of GV	No. UnInfected	% mortality
Granulovirus	0	19	31	28
Polyhedrovirus	20	0	30	26.2
aGV+NPV	20	13	17	66
bNPV.GV	23	15	12	76
cGV.NPV	20	19	1 1	78
Control	0	0	50	0
Treatment[a]	**Symptom of NPV**	**Symptom of GV**	**No. UnInfected**	**% mortality**
Granulovirus	0	10	40	19.2
Polyhedrovirus	14	0	36	27.5
aGV+NPV	29	14	7	83.6
bNPV.GV	21	10	19	61
cGV.NPV	35	14	1	92.3
Control	0	0	50	

A: For 2nd Instar
B: For 4th Instar

a GV and NPV were applied at the same time
b NPV **was** applied 24 hr perior to GV
c GV **was** applied 24 hr perior to NPV

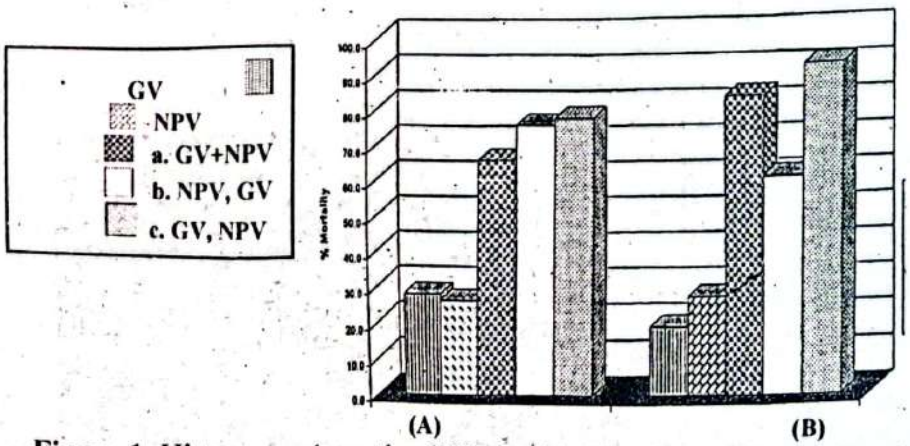


Figure 1. Histogram show the different % mortality of *in vivo* treatments by GV, NPV and mixed infections for 2 [nd] and 4 [th] instars:
a. GV and NPV were applied at the same time.
b. NPV was applied 24 hr prior to GV.
c. GV was applied 24 hr prior to NPV.

The resulted viral DNA characterization demonstrated that NPV was not changed the enzymatic restriction profiles when digested by EcoRI, EcoRV, HindIII, Bgl II, and Sal I. Also the SDS-PAGE in (fig.2) demonstrate the total expressed proteins of resulted viruses from different treatments these viruses which were differentiated, separated and purified as previously mentioned depending on the symptoms as showed in table (1), the protein profiles of the NPV purified from the mixed infection also the GVs were different from the comparable individual virus as demonstrated in fig.(2) lane 7 as supplemented band and missing or deleted band in lane 9.

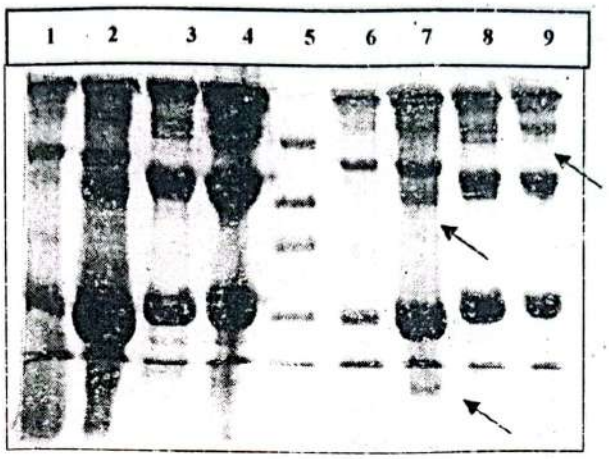


Figure 2. Demonstration of resulted viral protein profiles from in vivo infection from (1- 4) 10µl and from (6- 9) 5 µl were loaded in the gel : (1, 6) NPV, (2,7) NPV mix, (3, 8) GV, (4, 9) GV mix and (5) Marker.

As for the infection *in vitro* as showed in figure (3) infected cells which were daily followed up and photographed, cells which have cyopathic effect as polyhera formation (A) and/or vacuoles, granulations, and condensation of cells components (B) were categorized one category objected to hybridization test (fig.4) which confirm that the GV reaction intensity and replication in different cell lines was clearly better than the NPV infection.

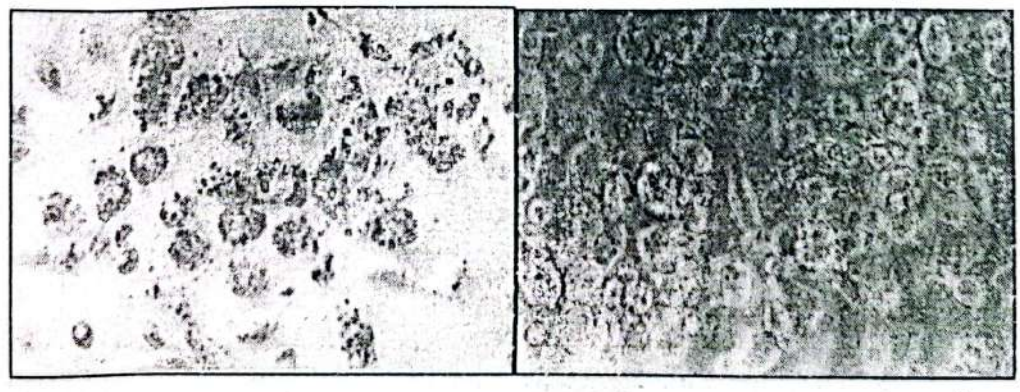


Figure 3. Micrograph demonstrates the cytopathological effects *in vitro* on Sl96 cell line infected by (A) NPV (B) mixed infection NPV-GV under the light phase contrast inverted microscope at 450X.

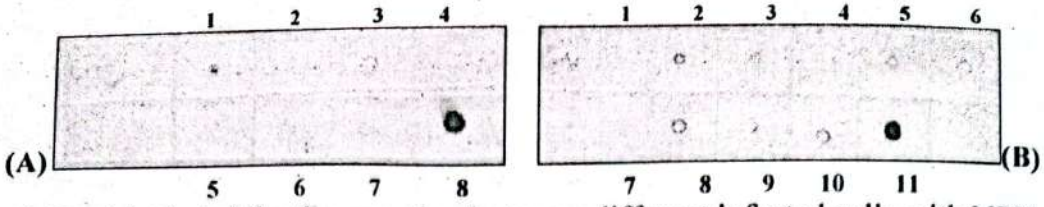
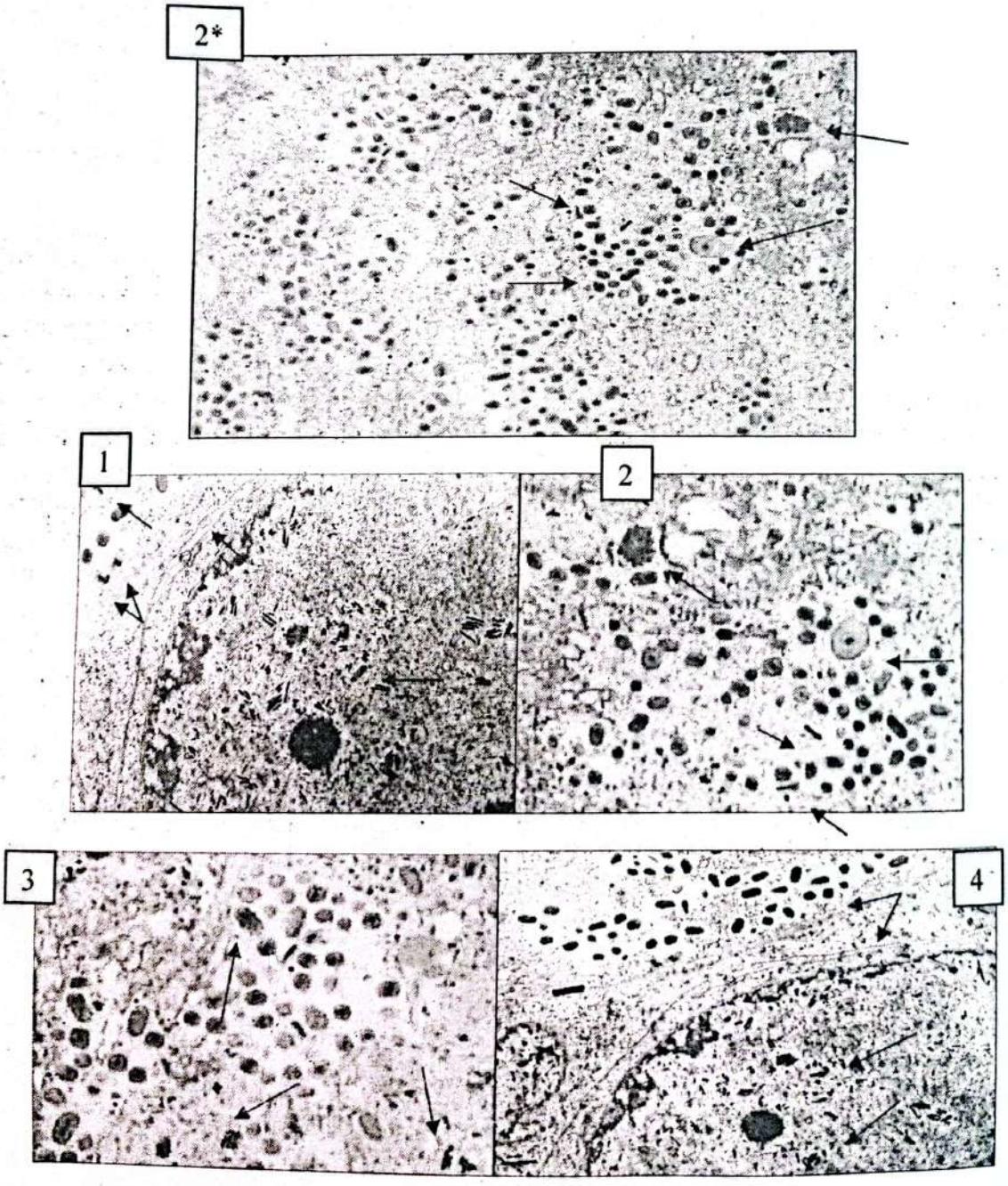


Figure 4. Dot blot hybridization reaction between different infected cells with NPV probe (A) and GV probe (B).

(A): NPV probe tested the treated cells:	
1-Sl 52 mix	2- Sl 96 Tr. Kf. GV M
3- Sf 9 mix.	4- Sl 96 NPV.
5- Sl 52 NPV.	6- Ld. NPV.
7- Sl 95 Tr. NPV.	8- Cont.+ AND NPV.

(B): GV probe tested the treated cells:	
1- Sl 95.	2- Sl 52 mix
3- Sl 95 GV MH.	4- Sl 96 Tr.Kf. GV MH
5- Sl 52 GV MH.	6- Sf 9 mix.
7- Ld. GV MH.	8- Sl 96 GV MH.
9- SF 9 GV MH.	10- Sl 96 GV MH.
11- Cont+ AND GV MH	



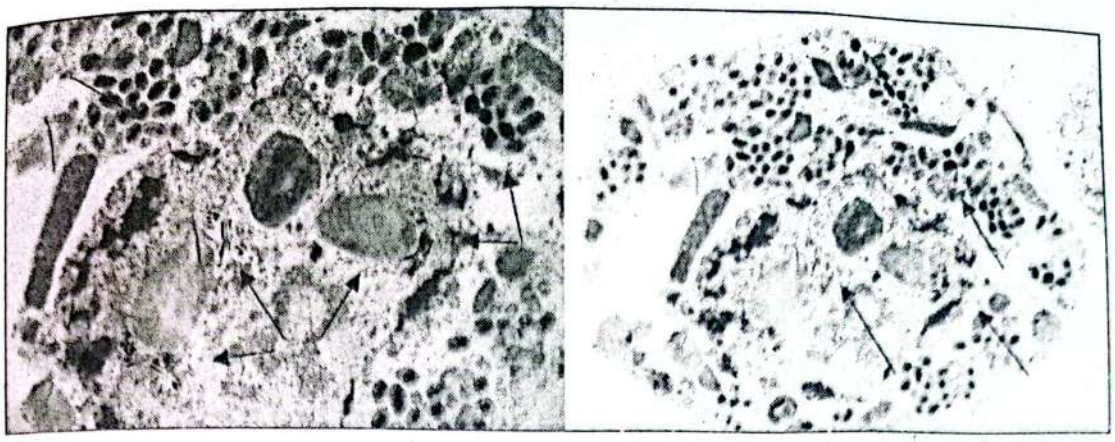


Figure 5. Electron micrographs representing SpliNPV and SpliGV replication morphogenesis and release 24 (1 and 4) and 72 (2, 2* and 3) h PI *in vivo* note the giant GV capsule at the same time of polyhedra formation and(5- 6) *in vitro* . Micrographs well demonstrate Virions which were seen budding through the plasma membrane (5 and 6), and the GVs complitly maturated they were observed free in the cytoplasm. The perinuclear space was enlarged, and NPVs virions were seen apparently budding through the inner nuclear membrane and the condensation of virogenic stroma in the peripheral of nuclear membrane. Well noted that maturated GVs existing only cytoplasm at the same time that NPVs still in very early stage of virions formation in the nucleous (5-6) *in vitro*.

These results were confirmed and agreed with the results obtained by the ultrathin sections of electron microscope as shown in figure (5) photos (5& 6) *in vitro*, electron micrographs representing the late stages of GV replication in cell cultures. By 36 h, the virogenic stroma of NPV in the nucleolus was dispersed; the GV granules were approaching their maximal size, and the nuclear membrane was still intact. Fibrillar material that had electron-dense material associated with it was seen also. Similarly in photos (2*, 2, 4) fig. (5) which represents the *in vivo* ultrathin sections, electron micrographs showed SpliNPV and SpliGV replication morphogenesis and release 48 (1 and 4) and 72 (2,2* and 3) h PI *in vivo*; micrographs well demonstrate virions which were seen budding through the plasma membrane, and the GVs completely assembled they were observed free in the cytoplasm. The peri-nuclear space was enlarged; NPV virions were seen apparently budding through the inner nuclear membrane and the condensation of virogenic stroma in the peripheral of nuclear membrane. Well noted that maturated GVs existing only in cytoplasm at the same time that NPVs still in very early stage of virions formation in the nucleous *in vitro* but *in vivo*, there was a spectacular formation of giant granulin (fig.5 (2) at the same field of polyhedra packaging of NPV (fig.5(2*and 2) and other GV virion during packaging surrounded by NPV virions in the nucleolus (fig.5(3,2)). These previous results in addition to the results of percentage of genome homology between the two tested viruses SpliNPV and SpliGV which were gave more than 60% homology when the GV was cut with EcoRI, it gave just 10.98% with Ac NPV (figure 6&table 2), the minimum % of homology was 24% with PstI when it gave 3.4% with Ac NPV,

Table (2): Represent the % of homology between GV-NPV.

REN GV	SpliNPV % Homol.	AcNPV % Homol.
EcoRV	36.45	8.82
EcoRI	60.44	10.98
PstI	24.60	3.15
Bgl II	27.86	36.51
Hind III	21.39	9.39

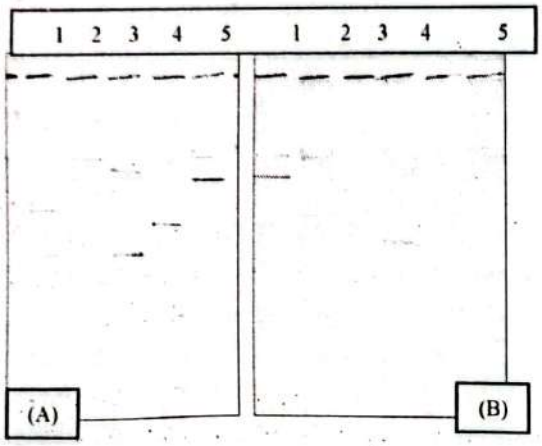


Figure 6. Hybridization gel transfer of digested GV by five restriction enzymes which demonstrated in table (2).
(A) Spli NPV probe and (B) Ac NPV.

DISCUSSION

The essential basis of these studies is better know complex interactions between baculoviruses infecting the pest in question (*Spodoptera littoralis*) in relation to stability or mechanism of infection in the same host. Results confirmed that mixing *in vivo* and *in vitro* reduced viral doses and reduced the time required for larval mortality that is much recommended for application in field. The results on table (1) which assume that mixing GV simultaneously or prior to NPV could agree with the hypothesis that **Van der Werf** *et al;* 2011 were discussed the interaction of baculoviruses in insects focused on mutualism and antagonism, even though the mechanism is not clear on mutualism. The antagonism of a Nucleopolyhedrovirus (NPV) with a Granulovirus (GV) has been extensively studied by a metalloprotein in the capsule of GV that disrupts the peritrophic membrane, the physical barrier to NPV entry to the midgut of larvae, to facilitate NPV infection. This could agree with the ultra thin sections results which demonstrate that despite the mixed activity or the final percentage of mortality was higher than each individual virus but the matured assembled virus was GV, the viral titer and new progeny was much more than NPV. So, it is probable that GV facilitate the penetration of NPV but then there is a

mechanism that enhances the GV to replicate and mature much more rapidly than NPV, but this explication could be *in vivo* depending on the statement or hypothesis of existing two physical phenotypes of viral particle occlusion-derived virus (ODV) and budded virus (BV). Both ODV and BV contain rod shaped nucleocapsids that are assembled within the nucleus **(Rohrmann, 2011)**, and as **(Harrison *et al.*, 2010)** explained that ODV are highly infectious to midgut epithelial cells and initiate the infection cycle in the animal. In contrast to the ODV, virions of the BV phenotype are generated earlier in the infection cycle, when nucleocapsids bud through the host cell plasma membrane into the hemocoel which could be the schedule for GV in our experiment that shown clearly in figure (5) 1, 2, 2* the assembling of GV particles a very few particle in the nucleus comparing with the matured particle in cytoplasm. This is in contrast with the NPV which is present in the nucleus only in few numbers of virions but apparently these ODV are highly infectious this expectation is agreed with the proposal that Clavijo *et al;* 2009 proposed when tested multiple genotypes of (SfNIC) NPV that infects *Spodoptera frugiperda* they concluded clearly that the NPV system fosters simultaneous co-infection by multiple genotypes, first by co-enveloping multiple nucleocapsids into ODVs and then by high multiplicity of infection of budded virions, each containing a single virus genome, within each infected insect, these could support the differences in protein profiles of mixed NPV–GV and giant GV in fig.(5). Also infection by multiple genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (Se-SP2) has been reported to influence pathogenicity of OBs (Mun˜oz et al 1999) and the virulence of the infection (Hodgson et al 2003; Horton and Burand1993) in NPVs of other species.

Multiple enveloping has the additional advantage that it accelerates the onset of infection as some nucleocapsids originating from the ODV are immediately rewrapped upon budding from primary infected cells to ensure establishment of systemic in-fection in the insect (Washburn *et al* 1999 and 2003) which is in my opinion could be the case in our work for the GVs which contain single nucleocapside in each virion, in addition to this could correlated with the type of fusion protein of each virus also (Khamiss *et al;* 2012) the size and diversity of baculovirus genomes appears to be strongly influenced by mobile DNA from the insect host. Also, transposon-mediated mutations of baculoviruses provide examples of functional inactivation of viral genes (FP phenotype mutations) and transcriptional activation (TE-D insertion (Blissard and Rohrmann 1990), another role transposable elements may play is the introduction of insect promoters and enhancers to the baculovirus genome. The importance of genotypic diversity and the interactions between genotypes in singly enveloped NPV or granulovirus populations is currently uncertain and need more very precise work by in situ hybridization and real time PCR.

Inaddition the heterogeneity in susceptibility is also a fundamental principle explaining patterns of pathogen genetic diversity among hosts in a population. What elements are indispensable in the description of the most basic host-pathogen interactions? Results drive to agree with Van der Werf *et al;* 2011 that variation in the host susceptibility of individual caterpillars to the virus is known to affect transmission of viruses between hosts, but it is also inextricably linked to infection biology and indispensable for understanding pathogen diversity in host populations.

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