THE FIRST DETECTION OF PINK BOLLWORM BACULOVIRUSES BY DNA NUCLEIC PROBE TEST IN EGYPT.

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INTRODUCTION

Baculoviruses (family Baculoviridae) are usually specific for one or a few insect species.The two subgroups of baculoviruses (*i.e.* Nuclear polyhedrosis viruses (NPV's) and Granulosis viruses (GV's) are considered as one of the most efficient bio-control agents for insects (Hunter-Fujita *et. al.*, 1998).

These viruses occur naturally and are present at low levels in many populations and can overwinter in the environment or in overwintering insects to re-establish infection in subsequent seasons (Hoffman and Fordsham 1993).

More than 600 baculoviruses have been reported in the literature and many have been tested for insect control of green house and field crops (Hunter -Fujita *et.al.*, 1998).

No threat to humans or wild life is posed by baculoviruses. NPV's have been registered in the United States for use against many caterpillars. GV's has been isolated from several caterpillar species (Hoffman and Fordsham, 1993).

The first record of viral infection was reported by Smith and Rivers (1956). Ignoffo and Adams (1966) proved the occurrence of a cytoplasmic polyhedrosis virus (CPV) infecting the mid gut epithelial cells of pink bollworm (PBW). Recently, a picorna -like virus, with a low rate of pathogenicity was identified and considered as a possible associated virus in the cotton pink bollworm larvae (Monsarrat *et.al;* 1995). Also PBW, *Pectinophora gossypiella* (Saunders) was susceptible to several viruses isolated from other hosts. So, *P.gossypiella* may be known as alternative host to certain lepidopterous entomoviruses (Monsarrat *et.al.*, 1995); in this regard, Vial *et.al.*, (1972) reported that, a nuclear polyhedrosis virus isolated from the alfalfa looper, *Autographa californica* (*Ac*NPV), infected larvae of the PBW, *P.gossypiella*.

In Egypt, Seufi and Osman (2005) isolated a NPV of *P. gossypiella* and evaluate its activity against 2nd instar larvae of *S.littoralis*. They found that, the LC 50

and LT 50 for the mentioned isolate were 4.1x 10^2 PIB/ml and 6.9 days, respectively. The present work was planned to record naturally occurring baculoviruses in *P. gossypiella* larvae collected from dried cotton bolls and double cotton seeds in Egypt. Identification of isolated baculoviruses from *P.gossypiella* took place in the "Center of Virology, Faculty of Agriculture, Cairo University, Egypt".

MATERIAL AND METHODS

I- Sources of isolates:

Samples of dry cotton bolls (Total collected 1000 bolls) and unheated cotton seeds (approximately 25 Kg) were collected from Qalubiya, Sharkia, Monifia and Fayoum Governorates during October - December 2003 and 2004. The alive resting pink bollworm (PBW) larvae were excluded from the mentioned samples and kept individually in clean glass tubes (2x4.5 cm) closed with plastic cover and left under natural room conditions .These larvae were observed for the appearance of viral disease symptoms previously recorded by El-Lebody (1998). Moribund larvae were kept in the freezer immediately after symptoms appearance. Figures of symptoms are presented after appendix.

II- Individual examination:

Moribund larvae showing distinct disease symptoms were individually examined for the presence of virus inclusion bodies, using light microscope. A wet smear of the homogenized liquid using a drop of haemolymph or a small part of larval tissue was spread on a clean slide. The slide was then dipped in 10% Giemsa's stain for 10 minutes. The excess stain was then washed with running water for 5 -10 seconds (Wigley, 1976). The prepared smear was examined using the oil immersion of phase contrast microscope. This smear test would allow recognition of the inclusion bodies of nuclear or cytoplasmic polyhedrosis viruses .The detection of new viruses was largely depending on the number of naturally diseased larvae collected from natural populations.

Larvae were macerated in distilled water using a suitable mortar. The resulting suspension was filtered through two layers of muslin to remove the undesirable fragments and skin, followed by centrifugation at 4000 rpm for 20 minutes (Bergold, 1974). Immediately, after centrifugation, the supernatant was stored while the pellet was examined in a smear test using light microscope for the presence of virus inclusion bodies of NPV and consequently determine the size of present isolated polyhedral inclusion bodies. The supernatant extract resulted from

4000 rpm was partially purified by centrifugation at 15,000 rpm for 20 minutes, for collection of granuolovirus.

III- DNA probe tests:

A DNA probe prepared from the total extract DNA of the *Spodoptera littoralis* NPV *(Spli* NPV*)* and *S. littoralis* granuloviurs *(Spil* GV*)* was used. The digoxygenin- labelled DNA probe was applied according to the protocol recommended by the supplier (Boehringer) (El-Hefny *et. al.,* 2000).

The DNA probe tests were prepared in the "Center of Virology", Faculty of Agriculture, Cairo University, Egypt."

IV- Laboratory rearing of PBW:

Neonate larvae of PBW (Lab. strain) were obtained from the Bollworm Research Division (PPRI), ARC and reared individually in glass tubes (2^x7cm) containing semi- artificial diet described by Rashad and Ammar (1984). The tubes were plugged with absorbent cotton and incubated at 26 ± 1C° and 80% R.H., larvae were allowed to feed on the diet. After about 10-11 days, these larvae were used in virus's propagation.

V- Virus isolate and propagation:

Viruses were purified from" naturally infected larvae of *P.gossypiella.* These viral isolates were propagated in laboratory reared *P. gossypiella* larvae (aged 10-11 days). The test was undertaken by surface - contamination of a small disc of 1 cm of the diet with the tested inocula (5 microlitre) (without the antimicrobial agent's formaldehyde methyl -p- hydroxy benzoate and sorbic acid), at the highest possible concentration. The contaminated diet was poured into glass tubes and offered to individual larvae aged 10-11 days starved for 6 hours. After 2 days of feeding on contaminated diet, all tested larvae were further maintained on clean diet for the occurrence of any symptoms similar to that of natural virus infection. The symptomatic larvae were observed after 5-7 days of treatment.

RESULTS AND DISCUSSION

The present study indicated that, the baculoviruses contaning a granulosis virus and a nucleopolyhedrosis virus of *Pectinophora gossypiella* are originally isolated from resting larvae of pink bollworm (PBW), *P.gossypilla* and recorded for the first time in Egypt, while their host ranges are unknown so far.

I- Symptomology:

The accomplished work indicates the following important points:

1- PBW resting larvae which did not exhibit symptoms of viral infection during early resting period treated with the baculovirus isolates; these larvae were normal in appearance, color and size. This result agrees partially with Hamm and Poschke (1963) who reported that, the cabbage looper larvae infected with GV often reach the size of normal full - grown larvae. Some of resting larvae of PBW incubated virus within their body were normal in appearance early and during the resting period (El-Lebody 1998).

2-The inoculated resting larvae of *P. gossypiella* exhibited the symptoms of viral infection at the end of the resting period. The symptomatic larvae often fail to pupate and die within 3 to 7 days later. This result agrees fully with El -Lebody (1998). In addition, the period from symptoms appearance to death may increase to 10 - 15 days if infected larvae reach pupal stage. The period from infection by a baculovirus to death of diseased larvae may vary and is affected by many factors, including larval stage, temperature, virus dose, virulence of virus isolate and nutrition of the larval host (Huger, 1963).

3-If symptomatic larvae reach pupal stage, the resulting pupae may be symptomatic or asymptomatic and few of these pupae may reach adult stage. In this respect , Houston (1991) reported that, low titers of the virus allow more hidden infection to occur because the infection are at low enough levels not to overwhelm the insect and cause death . Also, she noted that, a number of *H. zea* pupae that appeared normal were found to be inapparently infected with iridescent virus (HIV).

The present paper records the symptoms exhibited by resting *P. gosspiella* larvae and pupae infected with the isolated baculoviruses in the following sequence of stages:

Stage A:

The healthy PBW larvae have normal color and size (Fig.1).

A. l- The diseased larvae exhibit normal color (pink or yellowish pink) and size but the body becomes flaccid and the larvae can't move.

A.2- Diseased larvae are usually spindle shaped and tabers towards the tail and tabers slightly from the middle towards the tail and also towards the head (Fig.2).

A.3- Infected larvae exhibit a swollen appearance and the thoracic legs take a direction towards the head (Fig.3).

A. 4- The integument color of infected larvae become gradually opaque and may take brownish pink color and sometimes striped with white filament (Fig.4).

A. 5- Black spots may appear on the integument (dorsally and /or ventrally) of some diseased larvae (Fig.5).

A.6- Rarely, a white dorsal - lateral tumor extend interiorly from 6^{th} to 3^{rd} abdominal segments. This tumor darkens slowly and increases in its size (Fig.6).

Stage B:

B. 1- Ventrally, the internal tissues in the region from metathorax to the fourth abdominal segment are liquefied taking a yellowish white color (Fig.7A).

B. 2- The former liquefaction and yellowish color extend posteriorly to the 6^{th}abdominal segment; increase in size and the remained body of larvae may initially turn very dark brown and harden (Fig.7B).

B. 3- Sometimes, a thin white layer may appear on the integument (Fig. 8).

Stage C:

Often in this stage, the infected larvae die either as larvae or as a larval - pupal intermediate stage. On the other hand, some diseased larvae reach the pupal stage.

However, the symptoms of this stage included many detrimental effects as follows:

Ventrally, the skin of larvae or larval - pupal intermediate from metathorax to 4^{th} or 6^{th} abdominal segment becomes ruptured (Fig. 9). On the other hand, the larval death may occur without rupture of skin. In this case, a dermal cap was noted extending dorsally over the larval thorax to head. In addition, the posterior of larval abdomen is surrounded by white filaments (Fig. 10).

Stage D:

The infected larvae may not die and reach pupal stage. In this case, the resulting pupae may be symptomatic or asymptomatic.

The symptomatic pupae exhibit the following deformations:

D. 1- The pupal body appears short and small in size. Moreover, the last larval skin is hanging with the pupal body (Fig.12). In comparison, the healthy pupae are disclosed by casting the last larval skin (Fig 11).

D. 2-The diseased pupal body appeared swollen with an irregular oval shape (Fig.13).

Fig.(1): Nnormal color and size of PBW larva.

PBW larva exhibiting the following external disease symptoms due to infection with baculoviruses: (Stage A)

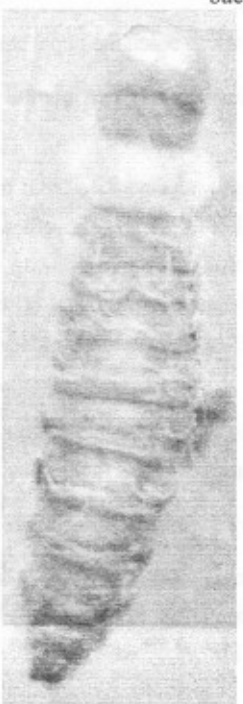

Fig. (2): Spindle shape, pink, brownish pink and brownish color.

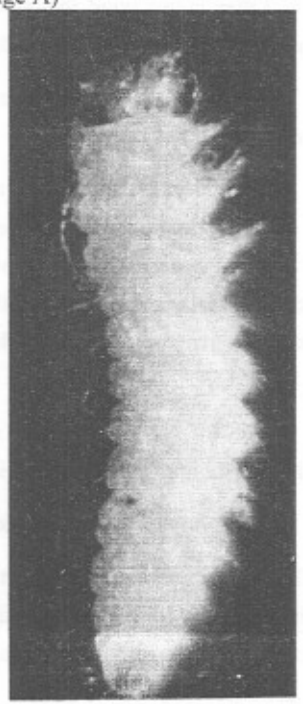

Fig. (3): Swollen appearance and thorax legs directed towards the head.

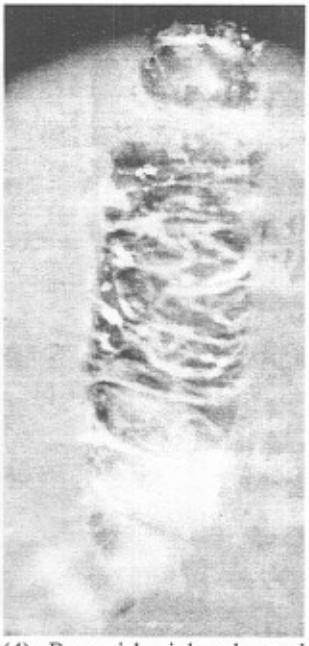

Fig. (4): Brownish pink color and white filament striped integument.

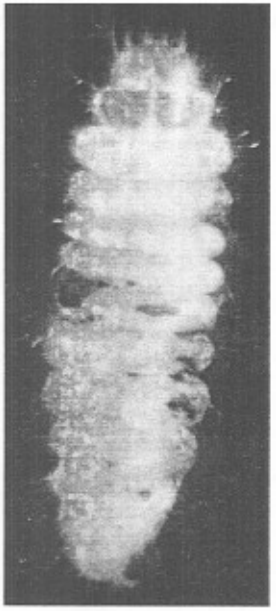

Fig. (5): Swollen appearance, opaque pink color, with dorsally and / or ventrally spots.

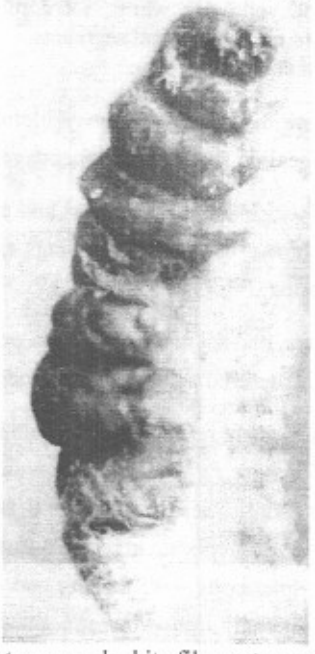

Fig. (6): Dorsal-laterally tumor and white filament surrounded the abdomen end

PBW larvae exhibiting the following external disease symptoms due to infection with baculoviruses : (Stage B)

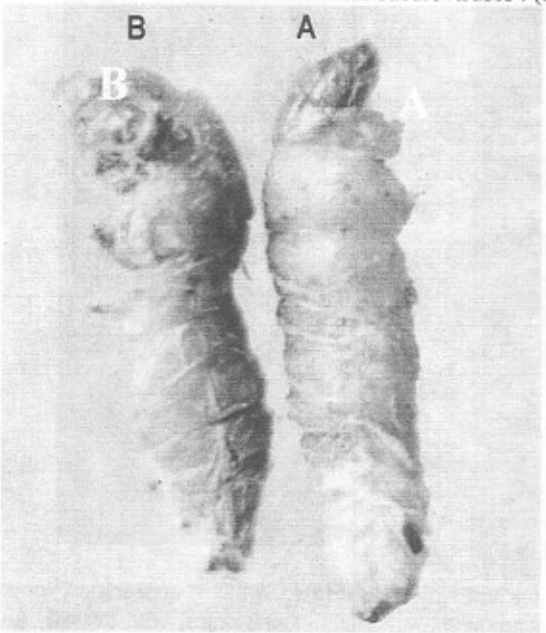

Fig. (7): A. Dorsally pink color, ventrally liquefaction and yellowish white in color of metathorax- 4 or 6 abdominal segments. B. Liquefaction and deformation.

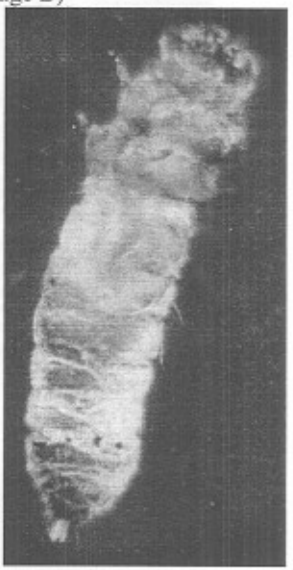

Fig. (8): Liquefaction, dark brown and thin layer on the integument.

D. 3- The surface of some diseased pupae may blotch with black spots and the antennae are not concealing as the other appendages to the pupal body (Fig.14).

D. 4- The anterior pair of wings is wrinkled, short and take dark brown (Fig.15 A & B) instead of shiny brown color; straight wings extend to the fifth abdominal segment of healthy pupa (Fig.11).

D.5- The symptomatic pupa, and asymptomatic pupa exhibit liquefaction of internal tissues and the skin is ruptured ventrally at the end of the disease cycle

Depending on the present results, it can concluded that, the viral infection of *P. gossypiella* with *PgGV* and / or *PgNPV* affect the pink bollworm movement, color , body content and development. These results agree with Hoffman and Fordsham (1993) who reported that, viruses replicate in many tissues and can disrupt components of an insect's physiology, interfering with feeding and movement. They added that, NPV infected larvae may initially turn white and granular or very dark .Victims of granulosis virus may turn milky white. In both cases, the body contents of the dead larvae are liquefied and the cuticle raptures easily.

PBW larvae exhibiting the following external disease symptoms due to infection with baculoviruses : (Stage C)

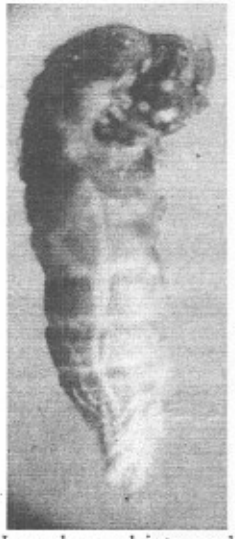

Fig. (9): Larval–pupal intermediate stage dark brown, hardness and skin rupture (ventrally).

Fig. (10): Dermal cap on the head & thorax and white filament surrounding of end abdomen.

II- DNA probe test:

Individual examination by phase contrast microscope of smears from naturally diseased larvae revealed the presence of virus particles. The inocula were recovered from purified suspension at 4000 rpm and 15,000 rpm pellet of NPV and GV, respectively (Fig.16).

The size of PIB's in the present isolate ranged between 1.06μm to 2.12 μm with an average of 1.65 μm (n= 40).

Naturally diseased larvae were subjected to further infection tests, using 10-11 days old larvae (as described in methods). The diseased larvae resulted from the re-infection test, recorded the same morphological symptoms of diseased larvae which collected from dry cotton bolls and unheated cotton seeds. In Egypt, the observed polyhedral inclusion bodies (IB) of PBW (5-12 □m in diameter) were recorded by Farrag (1976). El - Gemeiy (1983) gave a photo picture of the light microscope examination of smears of PBW diseased active larvae collected from green cotton bolls and thought to be of NPV. Also, she gave a figure of diseased larvae show one of the viral symptoms. Apart of that, NPV of *P. gossypiella* has never been recorded elsewhere in the world. *P. gossypiella* was represented by 3087 larvae collected from green cotton bolls, of which the rate of natural mortality was 31.1%. A wide

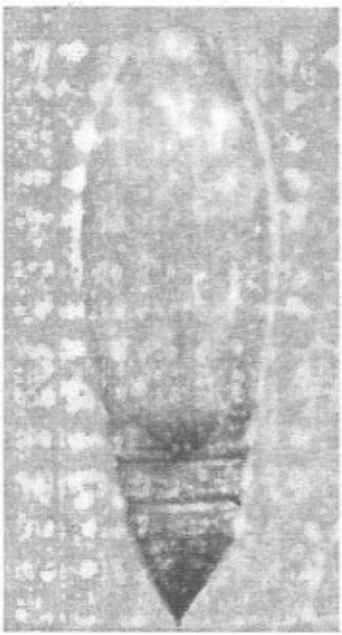

Fig.(11): Nnormal color and size of PBW pupa.

PBW Pupae exhibiting the following external disease symptoms due to infection with baculoviruses: (Stage D)

Fig. (12): Small and short appearance and the last larval skin hanging to pupal body.

Fig. (13): Irregular oval shape, swollen appearance and the last larval skin hanging to pupal body.

Fig. (14): The antenna not concealed to the pupal body.

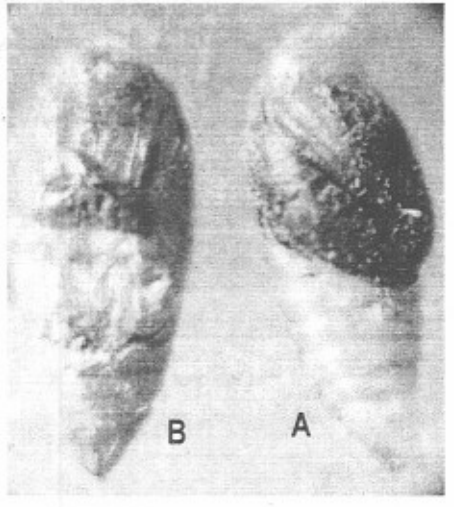

Fig. (15): A. Deformated interior wings. B. Skin rupturing ventrally.

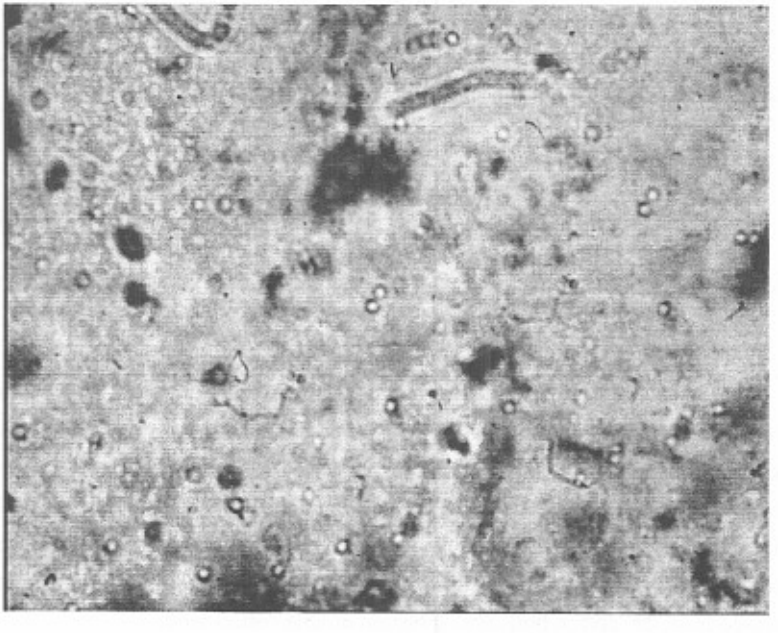

Fig.(16): Light microscopic examination of *P. gossypiella* purified suspension showing polyhedral inclusion bodies (1000X)

range of disease symptoms was observed, 9.4% explored larvae, 2.9% v-shaped, 5. 1% swollen bodied, 76.6% soft bodied and 3.6% dried larvae. Bacterial cells were frequently observed in 66.7% of dead larvae. The smear test of resulted dead larvae ; the infection test using suspect larvae carried out on the 3rd instar test larvae; and the electron microscope examination did not reveal any inclusion bodies (IB) (Khattab l988).

In the present study, two types of baculoviruses were detected in naturally diseased *P.gossypiella* larvae by probe test; a nuclear polyhedrosis virus (NPV) (Fig .15) and a granulosis virus (GV).

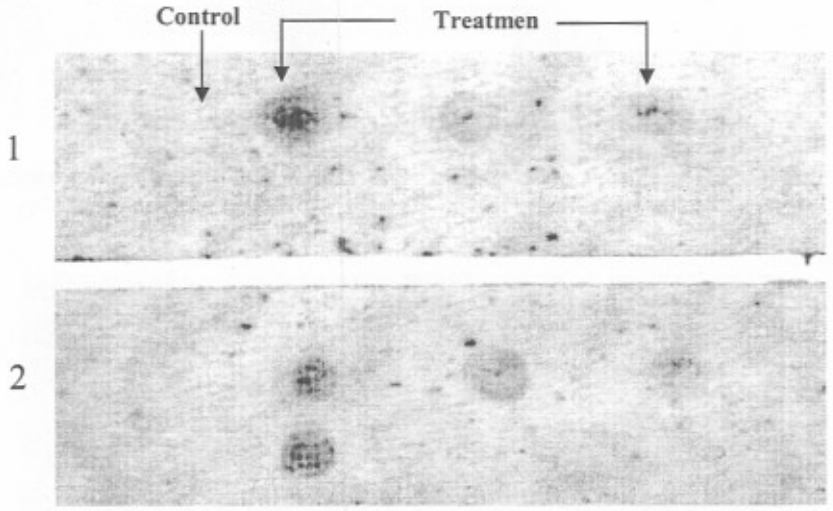

Fig. (17): DNA probe reaction on nitrocellulose membrane for the healthy (control) and diseased *P. gossypiella* larvae (Positive reaction indicated with samples using *S*LNPV (1) and *SL*GV (2) nucleic acid probes)

The dot blot test revealed relationships between *Spli* NPV DNA, *Spli* GV DNA with *P.gossypiella* purified suspension contained DNA. Also, a highly positive reaction (++) was observed between *Spli* NPV DNA and *P. gossypiella* suspension than *Spli* GV DNA (+). Thus, the DNA is a double stranded DNA of 137 Kb molecular weight (Fig 17). The DNA probe tests indicated the presence of *P. gossypiella* NPV and *P.gossypiella* GV in the *P.gossypiella* naturally infected larvae collected from dry cotton bolls and unheated double cotton seeds.

In conclusion, the low disease incidence was attributed to the natural low level of inocula. Also, further screening attempts are required as well as, special

restriction enzyme analysis.

III- The infection of PBW-laboratory reared larvae with *Pg*NPV and *Pg*GV isolates:

Primary tests of infection are conducted to observe the infection of 10-11 day old PBW larvae (lab - strain) with *Pg* GV and / or *Pg* NPV isolates. The observation indicated that, typical disease symptoms were exhibited by either laboratory PBW infected larvae with *Pg*GV and / or *Pg*NPV or naturally infected PBW larvae (natural population).This result indicates that, the present recorded symptoms were attributable to the viral infection of PBW with *Pg* GV and / or *Pg* NPV.However, the laboratory infected larvae showed viral symptoms after approximately 5-7 days of feeding on treated diet. This result agrees with Vial *et al.,* (1972) who reported that, the PBW larvae showed maximum symptoms after approximately 7 days of feeding on contaminated diet with *Ac* NPV. On the other hand, some treated larvae (Lab .strain) were asymptomatic and may survive and develop to adult. This result agrees with Houston (1991) who reported that, a number of *H. zea* pupae and adults that appeared normal were found to be in apparently infected with iridescent virus *(HIV)*. She added that, in many instances, the percentage of insects with inapparent infection was higher than or equal to those with apparent infection.

SUMMARY

Baculoviruses of Pink bollworm (PBW), *Pectinophora gossypiella* (*Pg*GV & *Pg*NPV) were originally isolated from symptomatic alive resting larvae in dried bolls and unheated double cotton seeds collected from various Egyptian Governorates during 2003 &2004. The DNA probe test showed positive reaction with *P.gossypiella* suspension using *Spli*GV and *Spli*NPV nucleic acid probes. Thus, the DNA probe testes indicated the presence *of P. gossypiella* GV (*Pg*GV) and *P.gossypiella* NPV (*Pg*NPV) for the first time in Egypt. The DNA genome is a double - stranded DNA with molecular weight of 137 kb. Present results pointed that, the host range of *Pg*GV and *Pg*NPV is unknown until now. In addition, the reinfection test using *Pg*GV or *Pg*NPV isolates against PBW (laboratory reared larvae aged 10-11 days) proved that, the present recorded symptoms were attributable to the naturally infection of natural population of PBW with *Pg*GV and *Pg*NPV.

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