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IN VITRO ISOLATION OF CLONES FROM THE TUNISIAN ISOLATE OF THE POTATO TUBER MOTH *(Phthorimaea operculella)* GRANULOSIS VIRUS

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ABSTRACT

Clones were obtained *in vitro*, as a first record, from the Tunisian isolate of the potato tuber moth granulosis virus (PTM GV). Using three successive slections with plaque agarose overlay, 12 clones were established. Among them, five were selected for their better *in vitro* multiplication after being tested with a specific DNA probe and amplified both *in vitro* and *in vivo*. Restriction endonuclease analysis of the DNA extract demonstrated minor genotype differences between the clones, indicating the genetic heterogeneity of the viral population of the Tunisian isolate of **PTMGV**.

Key words: Cell line, Granulosis virus, *Phthorimaea operculella,* Potato Tuber Moth, Viral clone.

1. INTRODUCTION

The baculovirus group, only infecting invertebrates, has always been of great interest. The essential knowledge on baculoviruses has involved Nucleopolyhedroviruses (NPV) for which susceptible cell lines have been selected and controlled (Goodwin *et al*., 1970). The absence of cell lines to suport replication for

granulosis virus (GV), the other important group of baculoviruses, has hindered progress on investigating their molecular biology and genetic manipulation.

There are few reports concerning negative or slight *in vitro* multiplication of GVS, (Miltenburger *et al.*, 1984; Granados *et al.*, 1986; Dwyer *et al.*, 1988), except for *Cydia pomonella* in which better multiplication occurred (Naser *et al.*, 1984; Winstanley & Crook, 1993). To reverse the situation regarding studies on Gvs, two conditions must be simultaneously met. First is the establishment of a stable *in vitro* GV multiplication to guarantee their permanent availability of the model; and second, the observation of a cytopahtological effect to permit cloning and genomic studies. Recently these two conditions have been obtained when a susceptible cell line to the GV from the potato tuber moth (PTM) *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) has been established (Léry *et al.*, 1995).

As the use of chemicals for the control of PTM, the main pest of potatoes in warm areas, is hazardous for humans and generates potatoes unfit for consumption (Von Arx *et al.*, 1987), this granulosis virus has already been applied in several countries (Matthiessen *et al.*, 1978; Raman *et al.*, 1987). As usually observed on NPV (Knell & Summers, 1981), the Gvs isolated from nature are constituted of genetically heterogeneous vairants (Arif *et al.* 1986; Crook *et al.*, 1985; Smith & Crook , 1988). Vickers *et al.* (1991), describing isolates of PTM GV from different countries, have demonstrated that several genotypes could be defined from those coming from Peru. For reasons associated with laboratory studies, safety tests and commercial production, it is desirable to be able to identify the virus before and after release into the field. The cloning of the granulosis viruses will lead to their essential step of identification.

In this paper, we report for the first time, the *in vitro* cloning of a granulosis virus and the preliminary characterization of five selected clones.

2. MATERIALS AND METHODS

2.1. Cell line:

The ORS-Pop-95 cell line (Pop 95) was used (Léry *et al.*, 1995). Cells between the 15^{th} and th 23^{rd} passage, were cultivated in Grace's modified medium (Léry & Fédière, 1990) containing 15% fetal bovine serum (FBS) and incubated at 19°c. The cells were routinely subcultured every 10 to 15 days and seeded with 1 to 2 x 10^6 cells per 25 cm^3 flask.

2.2. Virus isolate :

A granulosis virus isolated from the potato tuber moth *P. perculella* (Tunisian isolate) (PTM GV), provided by Dr. El -Bedewi (International Potato Center, Egypt) was multiplied in larvae reared in laboratory conditions and used for the cloning of the PT|M GV.

2.3. Cloning of the virus:

The viral suspension was prepared from infected larvae homogenized and ultrasonicated 1 min in 2 ml Grace's modified medium without FBS. After 2 centrifugations at 5,000g for 20 min and 15,000 g for 30 min, the supernatant was diluted in Grace's modified medium, and filtered on 0.45 um membrane. Four dilutions were used to infect 24h old cells cultivated in 35 mm Petri dishes at 19°c. After 4h contact at 19°c, the 0.5 ml viral suspension was removed. The petri dishes were layered on ice and the cells were over laid by a low melting agarose mixture at a final concentration of 1% containing Grace's modified medium with FBS, and maintained at 40° c. After 16 d incubation at 27°c. When gytopathological effect could be seen, the clones were picked up with a tapered Pasteur pipette, after lysis plates were revealed by staining with a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide (MTT) solution at 5 mg/ml in phosphate buffer saline (PBS) for 4h at 27°c. The collected clones were diluted in 0.5 ml Grace's modified medium wihtout FBS, then cloned twice again in the same conditions as above (Crook *et al* 1985;Granados *et al* 1986). The clones were then multiplied ; two weeks later, clones were purified and tested by DNA specific probe and the supernatant was

used to amplify the clones *in vitro*.

2.4. Amplification of the clones *in vitro*:

Three serial passages were performed to amplify the cloned viruses in the 19°c cell cultures into 25cm^3 tissue culture flasks which were inoculated with infectious supernatants of the clones and the infected cells were incubated at 27°c for 2 weeks

2.5. Virus purification from infected cells:

The PTM GV infected cells were collected 15 day post-infection to purify the virus by adapting the technique described by Granados *et al* 1986, scraped from the flasks and centrifuged for 5 min at 400 g. The pellet was treated in STE buffer 0.15 *M* Na Cl, 0.02 *M* Tris,1mM EDTA,05% Aprotinine and 0.5% NP4) for 10 min at 0 °C under agitation. The treated pellet and supernatant were homogenized with Potter and ultrasonicated. After centrifugation for 30 min at 15,000g, the pellet was resuspended in 0.01 *M* Tris (pH 7.5), deposited on a 30% to 70% (W/W) sucrose gradient and centrifuged for 20 min at 30,000g. The band containing granules was collected and concentrated as above, then resuspended in 10m*M* Tris, 1m*M* EDTA,pH8.0 (TE) and stored at -20°c. The GV concentration was determined with a spectrophotometer using the relationship OD 420nm 0.8=0.1 mg of granules/ml and 1mg granule/ml=6.0x10 ^{10} granules (Chang &Tanada, 1978). The supernatant was ultracentrifuged for 30 min at 100,000g. The pellet resuspended in Tris 0.01 *M* pH 7.5 overnight was deposited on a 20% to 50% (W/W) sucrose gradient and centrifuged for 1h at 100,000g. The band containing virions was collected and the particles were concentrated as above and stored at 20°c.

2.6. Amplification of the clones on *P. operculella* larvae:

Second and third instars of germ-free larvae, reared under laboratory conditions were fed on small potatoes (16g each for 25 larvae).The larvae were orally contaminated by dosing potato with a semi-purified granule suspension of the cloned virus produced *in vitro* The larvae were kept at 27°c plastic rearing boxes.

2.7. Virus purification from infected larvae:

After 7 to 10 days, white diseased larvae were collected and homogenized in 10m*M* Tris with Potter. After three cycles of clarification by sonification and centrifugation respectively at 800g for 5 min, 1,000g for 10 min and 1,250 for 20 min, the last supernatant was centrifuged at 15,000g for 30 min. The granules and virions were then purified as described for the cell cultures.

2.8. Nucleic probe detection:

Granules or virions purified from infected cells and cellular extracts were tested with DNA probe. One ml of infected cells and supernatant (1 to 2 $x10^6$ cells). were concentrated by centrifugation for 30 min in microfuge (13,000g). The pellet was resuspended in 100 ul TE, incubated for 30 min at 37C with an equal volume of 0.12 M Na2 Co3, pH10.9, then treated with 2% sarkosyl and proteinase k at a final concentration of 0.2mg/ml fot 2h at 50°c. The resulting suspension was used at different dilutions to be tested. The digoxygenim labeled DNA probe was applied according to the protocol recommended by the supplier. The same protocol was applied for the "dot blot" hybridization technique. A specific DNA probe prepared for a cloned fragment of the PTM was used (Unpublished data.)

2.9. DNA extraction and analysis:

DNA was extracted from purified granules according to the method of Summers & Smith (1987). The DNA was resuspended in TE to be used for endonuclease digestions and its concentration was determined by the spectrophotometer at 260 nm. wave length. Viral DNA (1-2ug) was digested for 2 h with 9 restriction endonucleases: Hind III, Eco RI, Bam HI, Eco RV, Sma I, BglII, Xho I, Pst I, and SalI, using the conditions recommended by the supliers (Boehringer). Electrophoresis of genome fragments for the comparison between clones and Tunisian isolate DNAs, was carried out on 0.75 and 1% agarose gel in 80mM Tris, 80mM phosphoric acid, 2 mM EDTA, pH8.0 (TEP), run at 60 V for 2h. The gels were visualized and

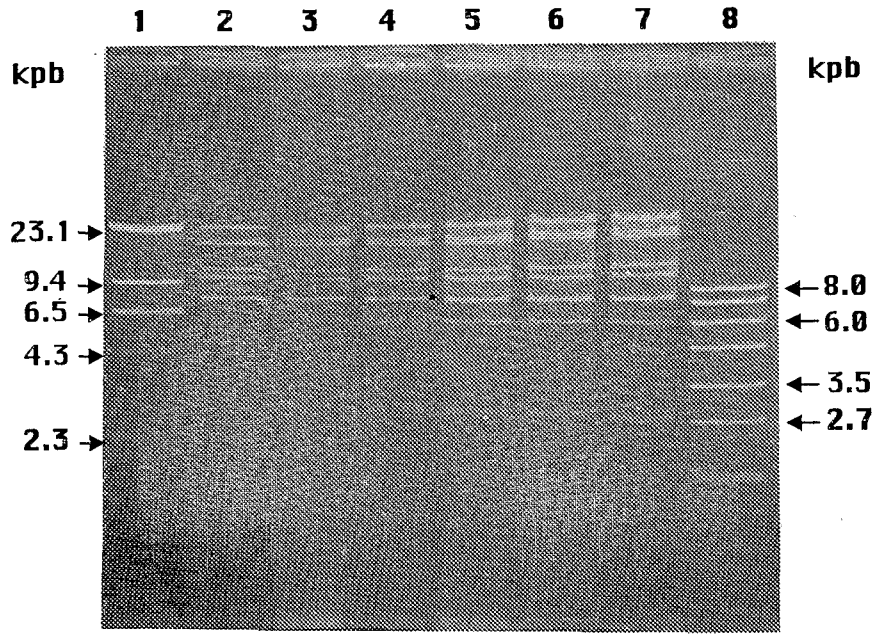
photographed on a UV illuminator. The size of the fragments was estimated by comparison with DNA from three succesive assays.

3. RESULTS AND DISCUSSION

Following the thrid cycle of selection by plaque assay, 12 clones of PTM-GV were obtained and tested using a specific DNA probe. Five clones were selected for their greater respsonse to be amplified *in vitro*. After three serial passages of these clones, small amounts of granules were obtained after purification. For this reason, the clones were also amplified *in vivo* on healthy laboratory- reared larvae of *P. operculella* to obtain sufficient amount of DNA for further characterization by restriction endonucleases. The DNA extract from the clones produced *in vitro* was only used to confirm the results obtained with enzymes which gave significant differences between the tested clones.

The results obtained by restriction enzymes, emphasized three different profiles in the 5 clones slected for their *in vitro* pathogenicity. Each clone was digested with a total of 9 restriction endonucleases (Bam HI, Eco RI, Hind III, Eco RV, Xho I, SmaI, PstI, Sal I, and BgI II) and compared with the wild type. Two of these enzymes, Bam HI and SmaI demonstrated differences among the clones With Bam HI (Fig.1), 2 profiles were obtained, 4.4 clones and 6.1 gave the first profile and clones 4.2, 8.1 and 8.2 gave the second one, with the 8.5 kbp fragment missing. All were different than the wild isolate PTM GV because of the lack or the slightly represented 9.4 kbp fragment and the presence of an intense 18 kbp fragment. With Sma I (Fig.2), the clones 8.1 and 8.2 were identical and had the same profile as the wild isolate.; Clones 4.4 and 6.1 were identical, but could be differentiated from the 2 other clones by the lack of the upper fragment (35 kbp). Finally the clone 4.2 had a distinct profile with 4 fragments the upper one at 28 kbp.

These results demonstrated that the Tunisian isolate was probably a mixture of several variants, as suggested by Vickers *et al.* (1991). The minor differences obtained between clones are common when comparing isolates or variants in the other baculovirus group of



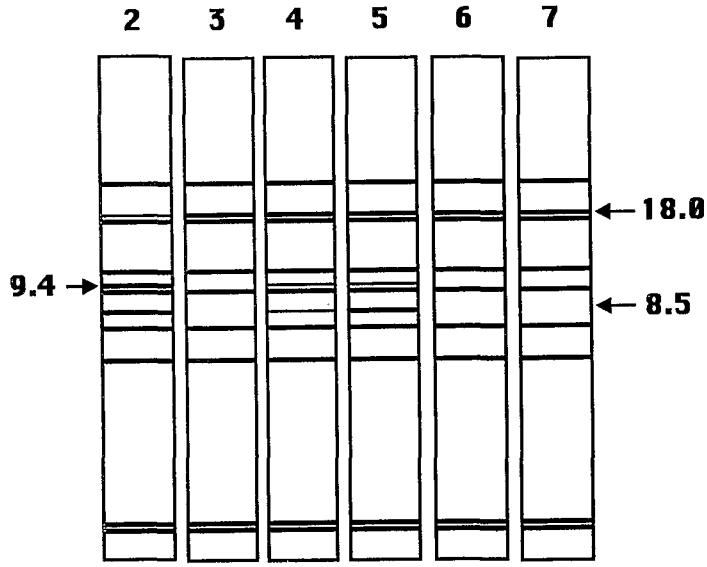


Fig. (1): Comparison of the electrophoretic profiles in 1% agarose gel of restricted genomic DNA from cloned and uncloned PTMGV, by Bam HI.

1: Marker;2: Wild type tunisian isolate; 3: Clone 4.4; 5: clone 6.1;6:clone 8.1; clone 8.2 and 8: marker.

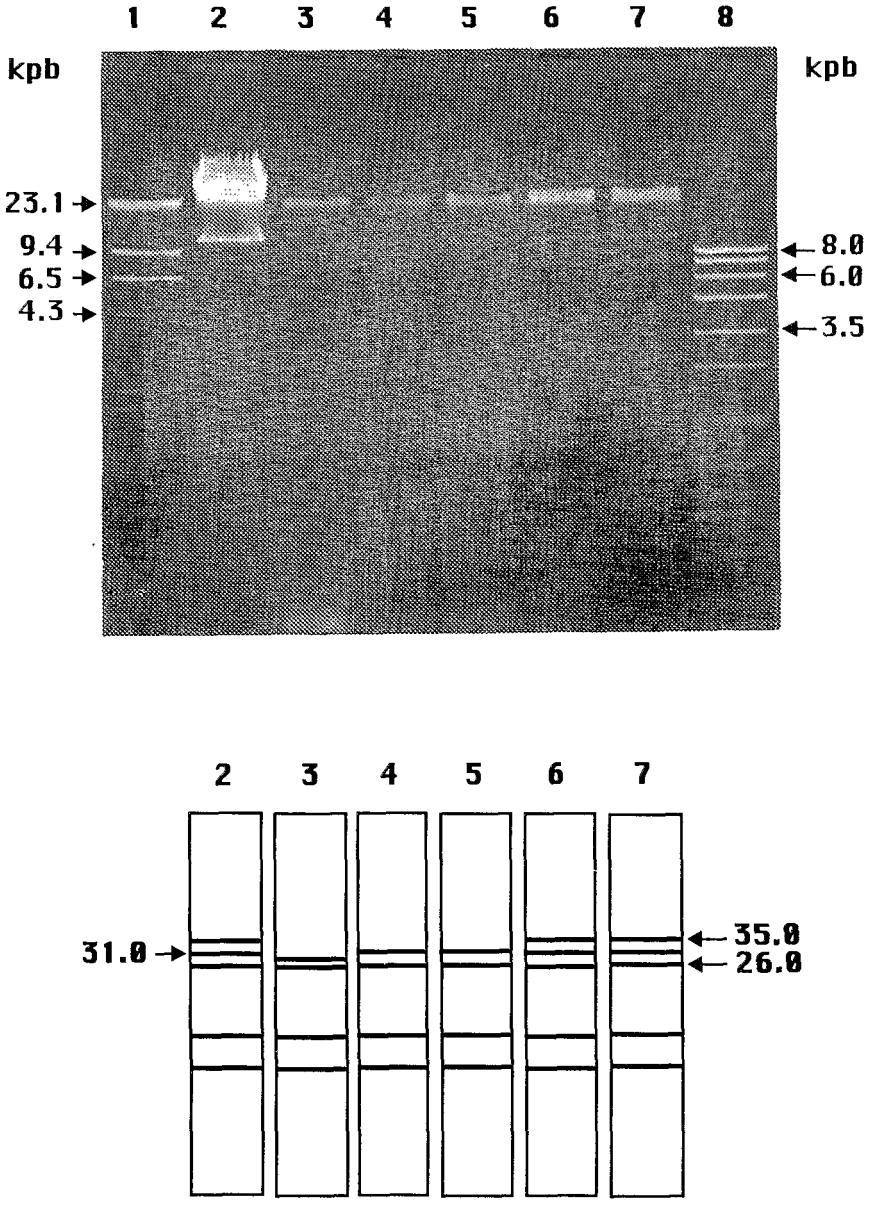


Fig. (2): Comparison of the electrophoretic profiles in 1% agarose gel of restricted genomic DNA from cloned and uncloned PTMGV, By Sma I.

1: Marker;2: Wild type tunisian isolate;3: clone 4.2;4: clone 4.4;5:clone6.1;6: clone 8.1; 7: clone8.2 and 8: Marker.

NPV (Crook *et al.*, 1985; . Smith & Crook, 1988). On PTM GV, the only study was conducted by Vickers *et al.* (1991), on eight geographic isolates.The results obtained by these authors concerning the Tunisian isolate indicated some small differences with the isolate used in our study, especially with the two enzymes which revealed differences between the clones. This indicated the heterogeneity of the viral populations of this isolate and probably that recombinations had occurred in Egypt as also obtaind on peruvian isolates of PTM-GV (Vickers *et al.*, 1991) or by several authors on other baculovirus models (Knell & Summers, 1981; Smith & Crook, 1988).

Depending on the clone, the quantity of granules produced varied from 1.82 to 5.0×10^9 granules/larva compared to the 3.0×10^9 granules/larva obtained when infection was performed with the Tunisian wild isolate produced first *in vitro* in the same conditions as the clones. This result confirmed that the *in vitro* multiplication of the PTM GV accurred at a relatively low level (Léry *et al.*, 1995). When larvae were infected by a viral suspension of 10^8 /ml the normal *in vivo* production could be around 2×10^{10} granules/ larva indicating that the inoculum used for the clones was lower than 10^8 /ml (Léry *et al.*, in press). Further tests will be performed to analyse the real impact of the different clones *in vivo*, but these first results could indicate that the 2 clones were 8.1 more pathogenic than the wild uncloned isolate. This possible conclusion was correlated with the previous results which demonstrated that small genotypic differences between closely related Gvs resulted in large differences in virulence (Crook, 1981; Harvey & Volkman, 1993). The same results were generally found on NPV models (Allaway & Payne, 1983; Cherry & Summers, 1985).

In conclusion, we have obtained, for the first time, clones of a granulosis virus *in vitro*. This work paves the way and forms the necessary baseline for further studies on GVs,especially on the PTM/PTM GV system. The importance of such studies is to follow the uncontrolled viral populations of GV already used in Egypt and several countries for biological control. In order to select the best clone for field trials and laboratory manipulations, the clones must be

compared in terms of virulence and of their *in vivo* and *in vitro* host range. So, the stability of the clones and the different isolates could be performed by the analysis of their genetic stability and the probable recombinations that may have occurred.

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أول عزل في مزارع الخلايا لكلونات فيروسية من السلالة التونسية لفيروس الجرانيولوسيز الممرض لفراشة درنات البطاطس

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ملخص

تم لأول مرة باستخدام مزارع الخلايا عزل كلونات فيروسية من السلالة التونسية لفيروس الجرانيولوسيز الممرض لفراشة درنات البطاطس. بتطبيق طريقة الاختيار المتتابع باستخدام شرائح الاجاروز، تم عزل أثني عشرة كلون فيروسي ومن هذا العدد تم اختيار خمسة كلونات تميزت بسرعة اكثارها في مزارع الخلايا وتم تشخيصها باستخدام المجس النووي المتخصص ثم اكثارها في مزارع الخلايا وفي الحشرات.

وباستخلاص الحامض النووي من الكلونات المذكورة بعد اكثارها كل علي حدة ثم تحليل هذه الاحماض النووية باستخدام انزيمات القطع لوحظت اختلافات في ترتيب تتابع النيوكليوتيدات مما يثبت الاختلافات الوراثية للكلونات المعزولة من العشيرة الفيروسية الواحد لسلالة الجرانيولوسيز التونسية الممرضة لفراشة درنات البطاطس.

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