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KARYOTYPING CHARACTERIZATION OF A CONTINUOUS NEW CELL LINE FROM PUPAE OVARIES OF EGYPTIAN COTTON LEAF WORM *Spodoptera littoralis* (SL-OMI) ADAPTED ON 27°C

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ABSTRACT

A new ovarian cell line was established from pupae of Egyptian cotton leaf worm *Spodoptera littoralis*, cells were adapted at 27C and characterized at the morphological, growth, and karyotyping . The number of chromosomes was counted and compared with reference cell lines Sf9, Sl96 and with the number of chromosomes on eggs (*in vivo*) of the same source of insect. Cell doubling time was 24hr. Chromosomes were morphological typical to those described from other species of Lepidopterous. Chromosomes number was counted on 57 metaphases for the new cell line (SL omi) and it was ranged between 4-203 Chromosomes. As for 58 metaphases were prepared from eggs, the counted chromosomal number was ranged between 4- 24 on 57 metaphases and one only metaphase had 46 chromosomes. Obtained results from eggs chromosomes preparation and Sl omi was different from those prepared from Sf9, which ranged between 61and 263 in 68 metaphase (Ref).

INTRODUCTION

The Egyptian cotton leaf worm *Spodoptera littoralis* is an important pest of several crops. Biological control of this pest with nucleopolyhedro virus of SL-NPV has been exploited (Okada, 1977). it nevertheless may be worthwhile to get continuous cell lines of this species it may provide a useful tool for the study of the virus as well as of insect cell physiology. Several cell lines have been established from the insects of the same genus; *S. frugiperda* (Vaughn *et al.*, 1977; Lynn and Oberlander, 1983), *S.exigua* (Getemter and Federici, 1986) and *S. ornithogalle* (Quhou *et al.*, 1984). In this study karyotyping was a first record for such study with this very important economic pest, and that is what lead to to compare the heterogeneity with the *Spodoptera littoralis* eggs, especially that when the modal number is close to the normal diploid number the variation about the mode is less when the modal number is greatly elevated (Valk.*et al*, 1996), beside there were some comparative studies with SF9 and SL96 reference cell lines (Mitsuhashi,1984).

MATERIALS AND METHODS

Primary culture:

Cells were originated from pupae obtained from the healthy insect rearing unit in the center of virology fac.of Agric.Cairo Univ. The method used for establishing primary cell cultures was derived from that of Volk *et al*, (1996). About 30 selected females pupae were surface sterilized by

immersion in 5% sodium hypochlorite for 10 min, two washes in ethyl alcohol 20%, and two washes in phosphate buffer saline (PBs; 0.137 MNacl, 5mMKcl, 5 m M glucose, 4 mM Na HCO3, PH 7.5) the pupae were dissected in ethyl alcohol 20% and ovaries collected, washed with PBs twice and cuted on very small pieces. Homogenized in a Teflon homogenizer, and then centrifuged for 5 min in buffer (2.6 mM KCl, 12mM NaHcO3, 137 mM NaCl, 0.4 mM NaH2PO4, 5.5 mM Glucose and 3.5 mM Citric acid .PH=7.2) Then centrifuged under the same conditions and the pellet was rinsed twice in PBS, the last pellet was re-suspended in Grace's modified medium (Lery and Fedeire, 1990) containing 20% of fetal bovine serum (FBS). Cells were seeded in Nunc tissue culture flasks (25cm2) with 4 ml medium and incubated at 27°C.

Sub culture:

During the first 4 months, 1 ml of fresh medium was added every 4 weeks .The first subculture was made after 4 months, Medium was added to the original one. From the fourth to the twelve passages the subcultures were done every ten days with the same selection techniques, after the twelve passage the cells were stored in liquid Nitrogen -196C and after two weeks cells were thawed and re-seeded with new fresh medium then sub-cultured.

Characterization of cells:

Growth curve: The morphology of cells was described and photographs of cultures were taken under normal light or with phase contrast inverted microscope at 100X to 500X, growth curves were determined from daily Haemocytometer counts of cells from two replicate cultures stained with Trypan blue over a period of 8 days. Cells were cultured in cell culture Petri dishes (35mm) seeded with 5.5x105 cells, the population doubling (PDT) time was calculated according to formula of Hayflik (1973); PDT= (t2-t1) log2/ (log n2-n1) where N1 is the initial cell number at select time t1 and N2 the final cell number at selected timeT2.

Karyology:

1- Karyotyping for cultured cells:

Karyotyping technique of obtained cell line was as follows: Cells were subcultured two days before harvesting the culture for chromosome spread., 0.1 ml of Colcemid solution was added to 3 ml of cell medium and incubated for 30 min at 27C, this incubation period was a result of several examination to detect the exactly favorable time. *Spodoptera littoralis* Cells were centrifuged at 1000 rpm for 8min and all supernatant was removed by suction apparatus but 0.2 ml of it. Cells were re-suspended in this small volume, 5ml of warm hypotonic solution were added and inverted to mix then incubated at 37C for 15 min. Cells were centrifuged at 1000 rpm for 8 min supernatant was removed but 0.2 ml of it, then, the cell pellet was re-suspended by gentle agitation without pipeting. 1 ml of cold fixative solution was added slowly, drop by drop with constant agitation, after 10 min another 4 ml of fixative was added and left on ice for 15 min., the fixation step was repeated twice as above. *(But the* fixative can be added more quickly on the 2nd and 3rd fixation) 15 min were allowed between successive fixation and cells were

stored over night at 4° C, cells were centrifuged at 1000 rpm for 8 min. Supernatant was removed and cells were suspended in about 0.5 ml of cold fresh fixative. Using a Pasteur pipette, 3-4 drops were dropped on a clean slide, besides blowing gently over the surface of the slide to spread the cell suspension thinly, The slide was put on hot plate for 5 min at 60 C to be dried thoroughly. Then slide was examined under the light microscope phase contrast so the adjustment can be made on subsequent slides if cells are too crowded or if spreading is poor. Slides were stored at room temperature in slotted trays or boxes without touching its surface. (Helle *et al.*, 1970, Gutierrez and Bolland, 1973 And Ronald G Worton and Catherine Duff, 1993)

2- Karyotyping of eggs using squash technique

For semi-permanent preparation one egg of 1-2 days old was soaked in a drop of 2% sodium citrate for 2-5 minutes on a clean slide. The treated egg with sodium citrate was soaked in aceto-orcin dye 1% for 10-15 min then heated on a flame and covered with a cover slide. After that the egg was squashed in aceto-orcin and a drop of the dye was passed under the cover slide then the squashed egg was examined with a direct or phase contrast microscope and photographs were taken from well metaphase plated. (Helle *et al.*, 1970 Gutierrez and Bolland, 1973).

RESULT AND DISCUSSION

1- Primary cultures

Cell migration occurred within eight months after the culture was set up. The migrated cells were either spherical shaped or rounded and spindle. They distributed themselves densely around explants at early stages, but gradually moved to the surrounding area and finally distributed over almost the entire area of the vessel (Fig.1,2).the spindle shape like of cells was seen at the beginning. The rate of mitosis in the migrated cells was initially very low, but gradually increased. In the fastest case, subculture becomes possible at 16 months after the initiation of the culture.

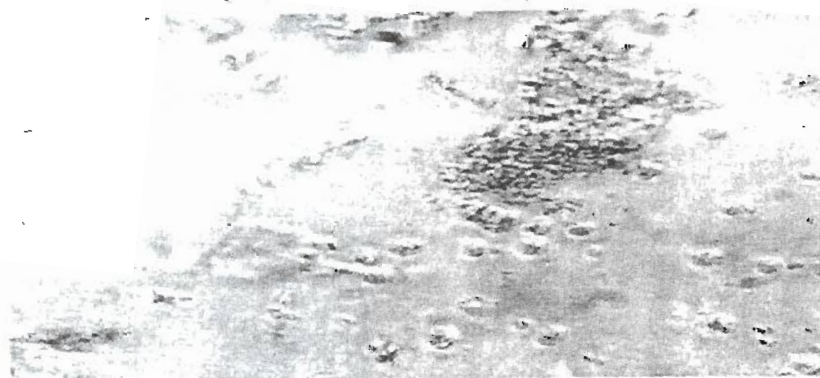


Fig (1): the cells in the primary culture

Fig (2): the cells in the primary culture, other field at the same flask

The growth of the cells in the primary cultures resembled that of the pupal ovaries of *M.brassicae* (Mitsuhashi, 1976) but only one modification was made is using the ethanol, as it was referenced before that using low concentrations of ethanol in insect cell cultures enhance the cell multiplicity ref, it was found that using ethanol with a final concentration of 20% at the establishment technique enhance the ability of cells in multiplication.

2- Sub culture:

Among over than 55 primary cultures prepared, four become possible to subculture, however, only one culture remained as a continuous subculture. The others terminated either by microbial contamination or deterioration. The first subculture of a continuous line was made after 16 months of the culture. At the confluent state of this culture, most cells attached to the substrate, however, the cells could be detached from the substrate by pipeting. the resulting cell suspension was divided into two vessels and an equal amount of fresh medium was added. Thereafter sub culturing were made in the same way (1: 2 split ratio) until the 60 passage till now. the growth of cells at early passages was slow, and the intervals between successive sub culturing were quite long. This cell growth becomes consistent after the 20th passage. The continuously growing cell line that was obtained was designated as SI-Omi.

3- Characteristics of SI-Omi cell line:

Morphology: The cells were all spherical at the beginning, and some spindle cells become established when cells become consistent at the 20th generation of the sub culturing. (Fig 3, 4), the volume of cells was homogenized.

Cellular adhesives: The cells showed a tendency to attach to the bottom of the culture vessels. However, the attachment was not very firm at the first generations, and the cells were easily to detach from the substrate by flushing the culture medium onto them from a pipette. By the course of time the cells attached to substrate initially become very attached to the flask. So the SI-Omi has changed this property within three months. Overgrown cells piled up, or detached from the substrate and floated

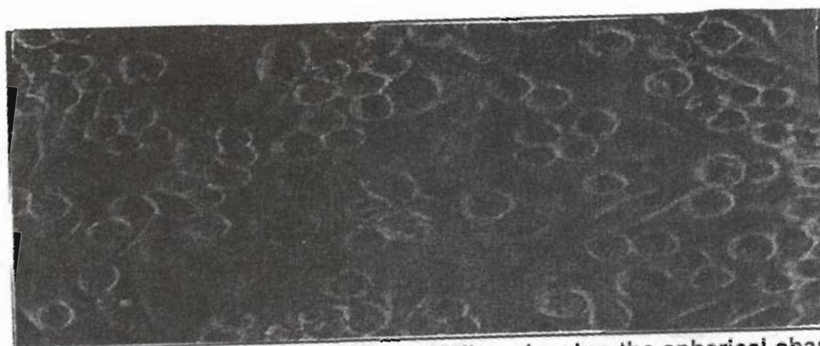


Fig (3):The population of the Sl-Omi cell line showing the spherical shape of the cells, and that some of them only are spindle. (40X).

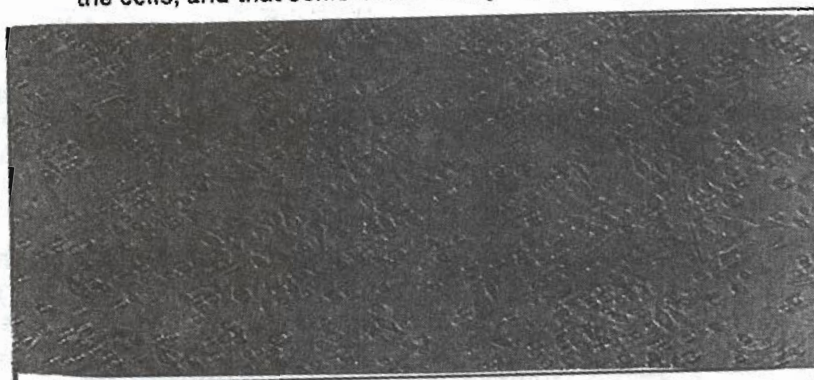


Fig (4):The population of the Sl-Omi cell line with the normal 10X.

Growth media and growth rate: Although the primary culture was initiated with Grace modified medium containing 20% fetal bovine serum (FBS) after the growth rate of the cells become consistent, the cells could be adapted to the same Grace modified medium but with 10% FBS only. Growth curve of the SL-Omi adapted at 28°C are shown in Fig (5) and table (1). The population doubling time was estimated to be 24hours.

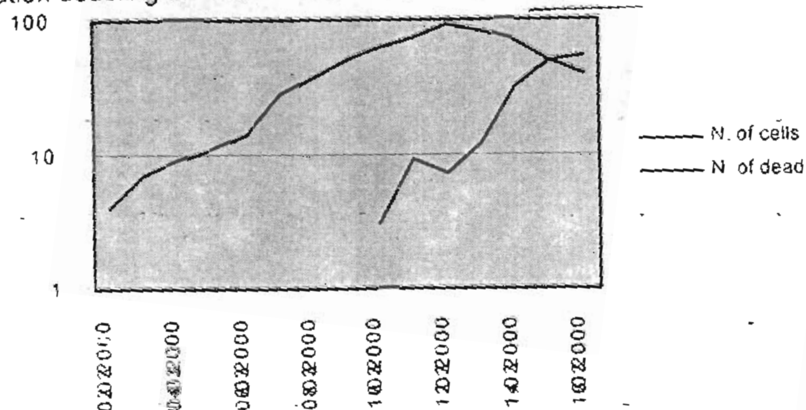


Fig (5): Growth curve of Sl-Omi cell line at 28c in Grace Modified medium with10%FBS.

Table(1): **The increasing of viability of the Sl-Omi Using trypan-blue.**

Days post zero time	N. of cells	Total no. /ml.melion	N. of dead	Total dead /ml.melion
3-Mar	4	0.04	0	0
4-Mar	7	0.07	0	0
5-Mar	9	0.09	0	0
6-Mar	11	0.11	0	0
7-Mar	14	0.14	0	0
8-Mar	28	0.28	0	0
9-Mar	38	0.38	0	0
10-Mar	52	0.52	0	0
11-Mar	63	0.63	3	0.03
12-Mar	73	0.73	9	0.09
13-Mar	91	0.91	7	0.07
14-Mar	82	0.82	12	0.12
15-Mar	70	0.7	32	0.32
16-Mar	50	0.5	50	0.5
17-Mar	40	0.4	55	0.55

Karyotyping: 1- The SL-Omi new cells Line: There was great diversity in the number of chromosomes. Majority of cells showed metaphase plate like Fig (6) each chromosome was very small and seemed to have a diffuse centromere; it was a typical metaphase plate for lepidopterous cell lines currently known (Mitsuhashi, 1981, 1983, 1984; Mitsuhashi and Shozawa, 1985; Mitsuhashi and Inoue, 1988; Inoue and Mitsuhashi, 1988).

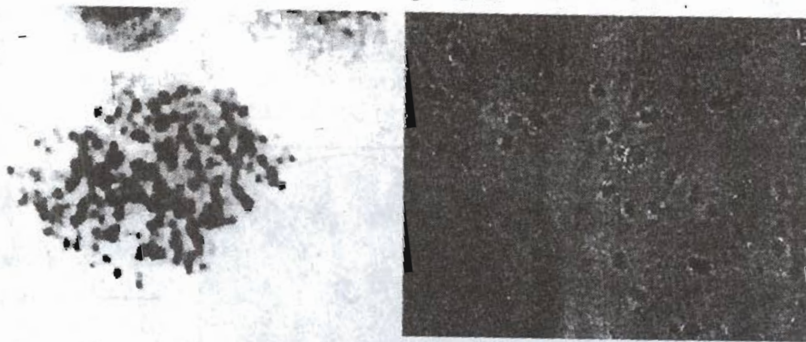


Fig (6):A- a typical metaphase of *Spodoptera litura* (TUAT-SpLi-221) cell LineJun Mitsuhashi 1994.

b. A typical Lepidopteron karyology of *Heliothis zea* embryos culture. (Mitsuhashi,1976).

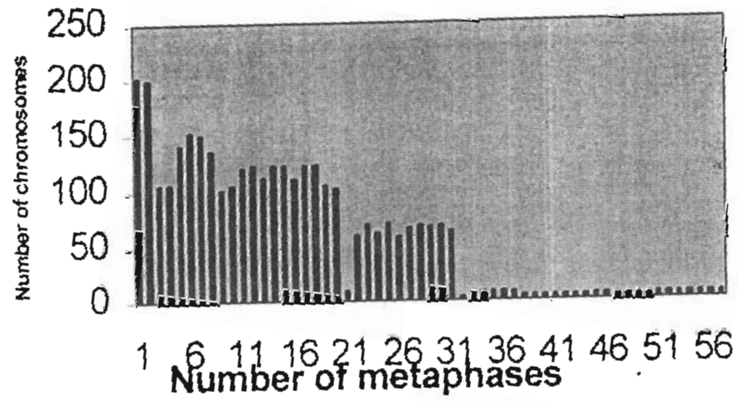


Fig (7): The number of chromosomes in the counted metaphases of the SI-Omi.

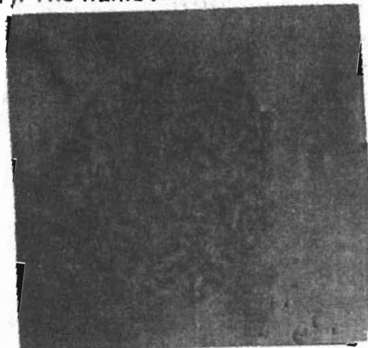


Fig (8, a)

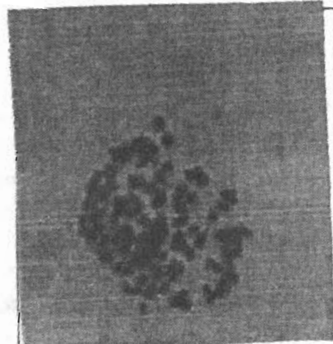


Fig (8, b)

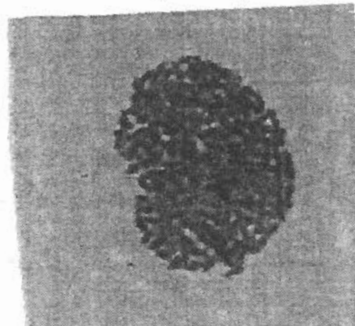


Fig (8, c)

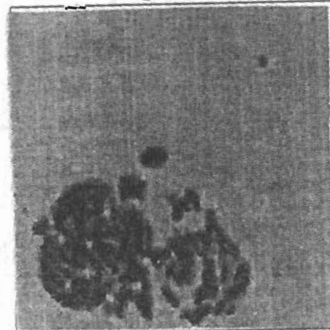


Fig (8, d)

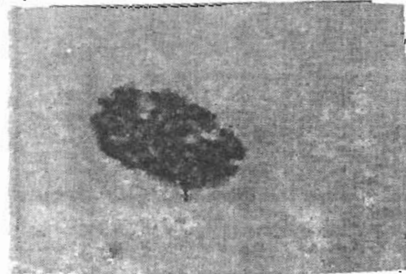


Fig (8, e)

Fig 8: the SI-Omi karyotype metaphases.

Karyotype for the initiated cell line was examined at the logarithmic phase of the culture, the colchicin was added to the culture medium so as to give the final concentration of 5x10 5 M. (J.Mitsuhashi, 1984) but The culture was kept for only half an hour not three hours as it referred to in the same reference because several treatments were made to detect the optimum time and the optimum concentration for this cell line and it found to be half an hour. The cells were collected and it was drop by drop mounted on clean cover glass and stained and finally examined, and the Chromosomes number was counted on 57 metaphase, it was obvious from the results that: there was a great diversity in the number of chromosomes, Majority of cells showed metaphase plate like (8, a-b-c and d) The average chromosome number was estimated to be around 103 and it was ranged between 4-203 chromosomes, as it shown in Table (2). 23% of counted metaphases contained 6 chromosomes only, 8% contained 12 chromosomes, 14% contained 8 chromosomes, 17% was in between 50-70 chro9mosome and 26% was about 100-120.2-The *Spodoptera littoralis* embryo karyotype: The only modification is the final concentration of colchicines was 10x 10 5 and the culture was kept for only half an hour, and the result was close to the new cell line more than the related reference cell line, and this was expected as it is reviewed in literature (Ronald G Worton and Catherine Duff, 1993) it was found that the range of chromosomally number was in between 4- 42 chromosomes, 17% of the 58 counted metaphases showed only 8 chromosomes, 15% showed 6 chromosomes, 12% showed 14 chromosomes and 10% showed 12 chromosomes only.

Table (2): The number of chromosomes in each metaphase in the new *Spodoptera littoralis* insect cell line:

Karyotype for *Spodoptera littoralis* New cell line					
Metaphase Number	Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number	Chromo. Number
1	203	21	10	41	5
2	200	22	60	42	5
3	106	23	69	43	5
4	105	24	62	44	5
5	141	25	70	45	6
6	153	26	58	46	6
7	150	27	66	47	6
8	136	28	68	48	6
9	101	29	67	49	6
10	104	30	68	50	6
11	120	31	63	51	6
12	122	32	8	52	6
13	112	33	8	53	6
14	122	34	8	54	6
15	123	35	8	55	6
16	111	36	8	56	6
17	123	37	8	57	6
18	123	38	5		
19	104	39	5		
20	102	40	5		

Table 3. and as it is obvious in the fig (9). The Majority of cells showed metaphase plate like, each chromosome was small but pigger than the new cell line metaphases and seems to have a diffuse Centromere, and that it was typical to lepidopteron cell lines currently known.

3- **The reference cell lines results:** The importance of this step is to indicate the homogeneity between the new and the old established cell line, and to put our hands on the fact wither the modal number become narrow at the old established cell line or not in the *Spodoptera littoralis* especially .3-1: SL 96 cell line: Karyotyping in New Sl96 cell line showed in 40 metaphases 42% from 50-70 chromosome and 17% of them ranged in between 90-124 chromosomes. Fig (10) and Table 4. 3-2: SF 9:Sf9cell line which showed chromosomal number range from 61- 263 in 68 metaphase 30% was ranged from 103-120 and 25% showed range from 50-70 chromosomes. Fig (11) and Table 5.

Table (3): The chromosomal number in 40 different metaphase in *Spodoptera littoralis* egg :

Karyotype for *Spodoptera littoralis* eggs					
Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number
6	41	24	21	7	1
6	42	5	22	11	2
6	43	5	23	15	3
6	44	5	24	46	4
6	45	5	25	4	5
6	46	5	26	4	6
6	47	12	27	4	7
6	48	12	28	10	8
8	49	12	29	10	9
8	50	12	30	10	10
8	51	12	31	18	11
8	52	12	32	18	12
8	53	14	33	18	13
8	54	14	34	16	14
8	55	14	35	16	15
8	56	14	36	16	16
8	57	14	37	16	17
8	58	14	38	24	18
		14	39	24	19
		6	40	24	20

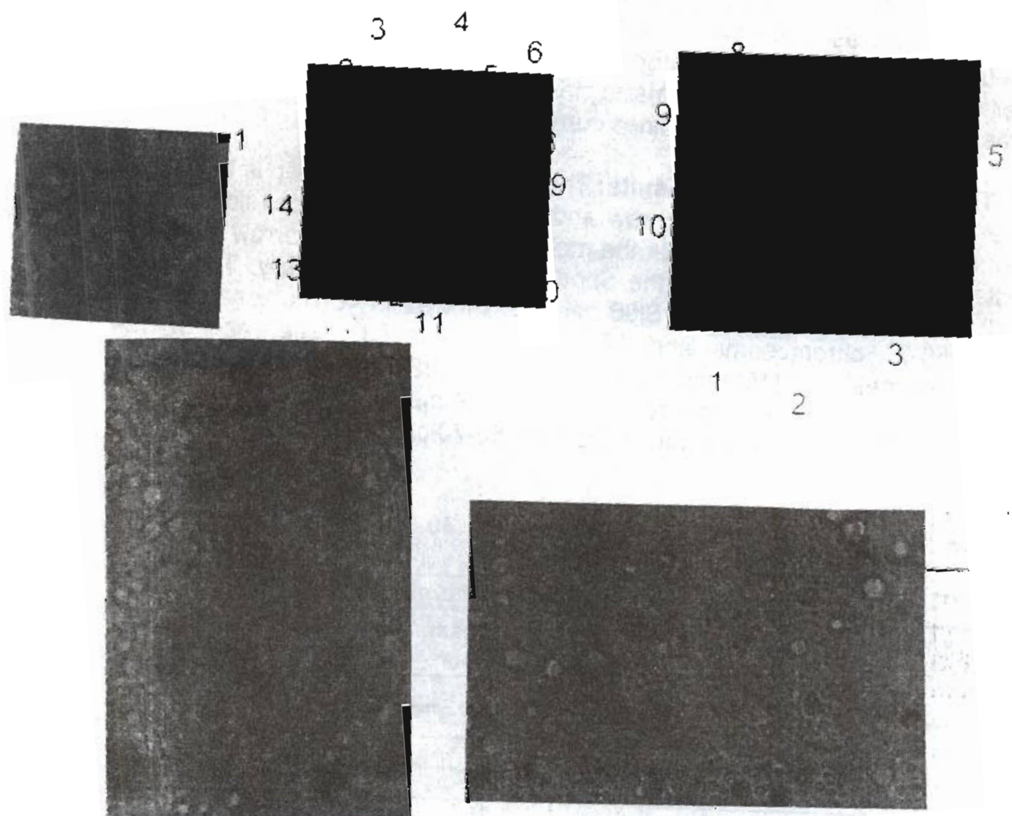
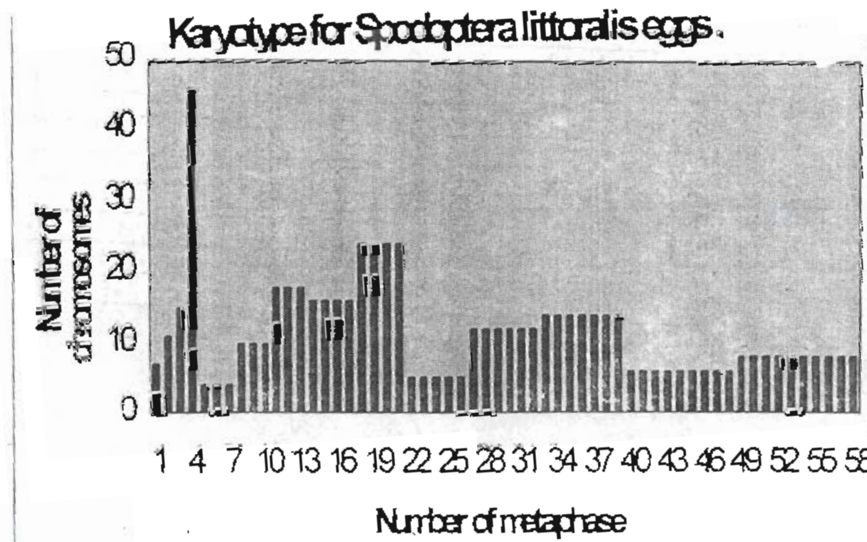


Fig (9): Different fields of egg of *Spodoptera littoralis*:



Fig(10) showing karyotype of *Spodoptera littoralis* egg:

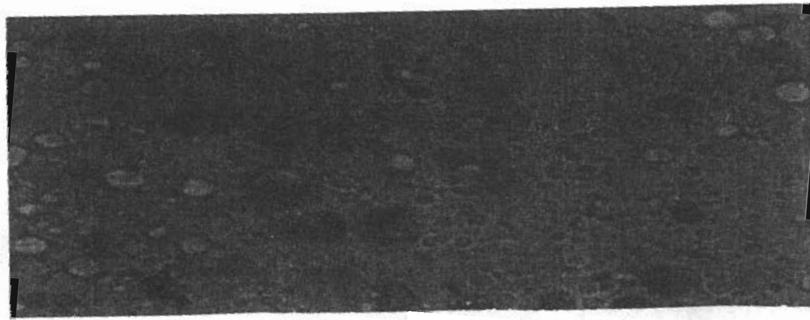
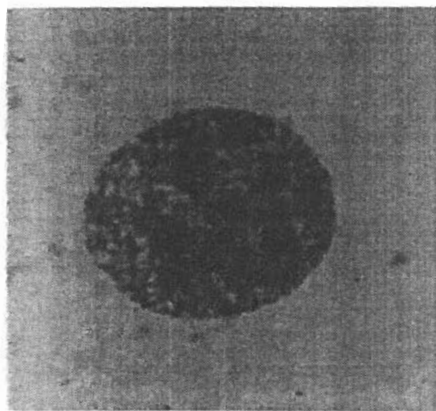
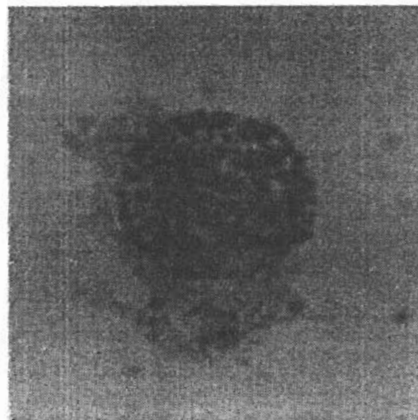


Fig (11): Complete field of different metaphases of *Spodoptera littoralis* egg:





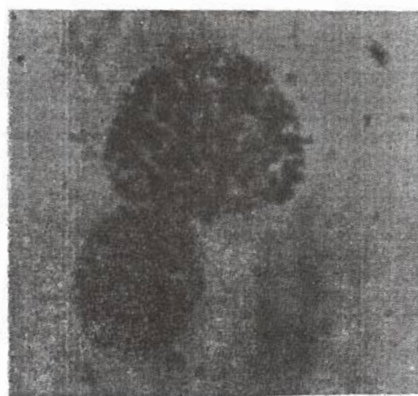


Fig. (11-a, b, c and d): Showing different metaphases of Sl96 as a reference cell line:

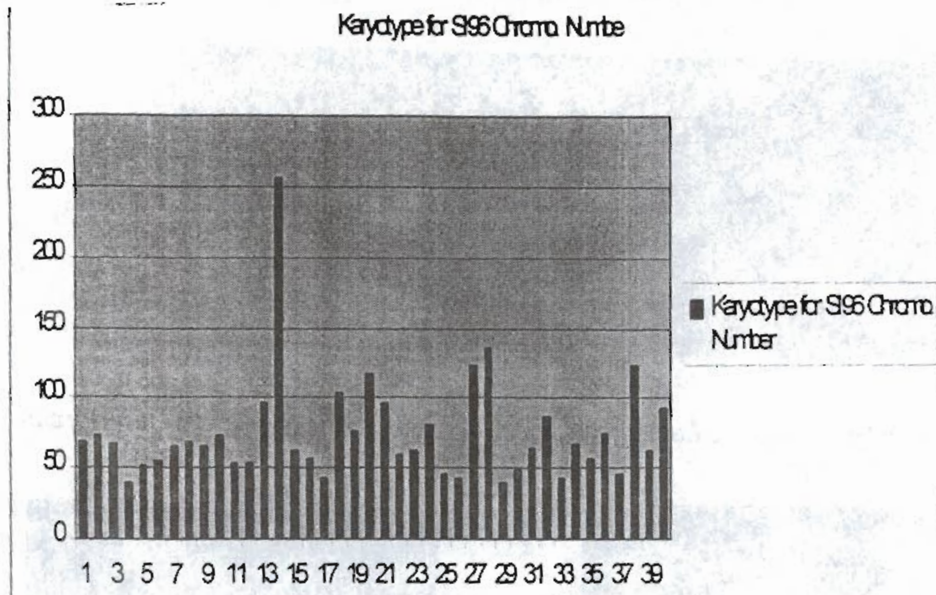


Fig (12): The number of chromosomes in different metaphases of SL96 reference cell line:

Table (4): The number of chromosomes in different metaphases of SL96 reference cell line:

Karyotype for Sl96			
Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number
96	21	69	1
60	22	73	2
62	23	67	3
80	24	40	4
46	25	52	5
42	26	55	6
124	27	66	7
136	28	68	8
40	29	66	9
48	30	73	10
64	31	54	11
87	32	53	12
43	33	96	13
67	34	256	14
56	35	63	15
74	36	57	16
45	37	42	17
124	38	103	18
62	39	76	19
93	40	118	20

Table (5): The number of chromosomes in different metaphases of Sf9 reference cell line:

Karyotype for Sf9 cell culture							
Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number	Chromo. Number	Metaphase Number
213	61	62	41	104	21	39	1
153	62	63	42	104	22	91	2
102	63	69	43	109	23	103	3
201	64	45	44	87	24	145	4
131	65	112	45	154	25	113	5
263	66	67	46	88	26	113	6
64	67	103	47	68	27	57	7
68	68	83	48	89	28	193	8
		121	49	56	29	120	9
		102	50	40	30	102	10
		67	51	61	31	145	11
		112	52	50	32	185	12
		143	53	62	33	65	13
		62	54	88	34	76	14
		93	55	236	35	114	15
		70	56	72	36	104	16
		91	57	102	37	89	17
		63	58	112	38	104	18
		106	59	70	39	276	19
		101	60	68	40	115	20

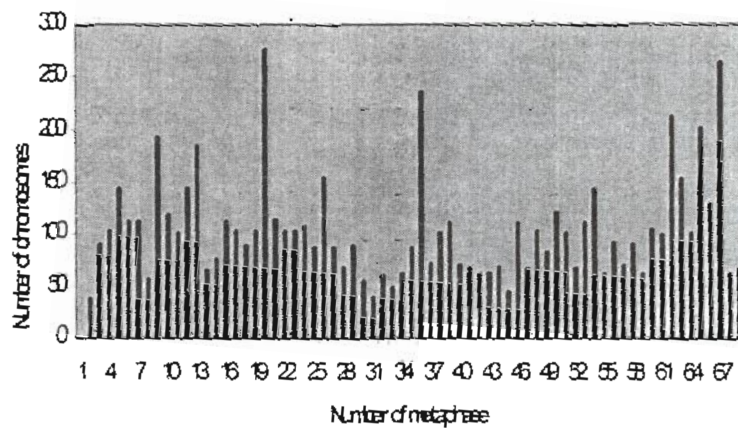


Fig (13): The number of chromosomes in different metaphases of Sf9 reference cell line:

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دراسة كروموسومية توصيفية لمزرعة خلوية مستمرة من عـذارى حشـرة دودة ورق القطن(Sl-Omi) منماة على ٢٧ °م

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تم انتاج مزرعة خلوية جديدة من عذارى حشرة دودة ورق القطن ، تم انماء هذة المزرعة على ٢٧ م و تم توصيف هذة المزرعة الخلوية طبقا للشكل المورفولوجى ،درجة النمو،و البصمة الكروموسومية.

الكروموسومات تم عدها و مقارنتها بالخلايا المرجعية، و بعدد الكروموسومات فى البيض الخاص بنفس الحشرة.

زمن التضاعف الخلوى وجـد انـة٢٤ سـاعة - و الكروموسـومات كانـت متطابقـة مورفولوجيا لتلك الموصوفة من الأنواع الأخرى التابعة لرتبـة حرشـفية الأجنحـة- تـم عـد الكروموسومات فى ٥٧ طور ميتافيزى بالنسبة للمزرعة الخلوية الجديدة و كانت متراوحة بين ٤-٢٥٣ كروموسوم بينما تم عد ٥٨ طور ميتافيزى للبيض و كانت الأعداد متراوحة بين ٤-٢٤ فى ٥٧ منهم و طور واحد فقط كان التعداد فيه ٤٦ كروموسوم، وكانت النتائج الخاصـة بالمزرعـة الخلوية الجديدة و البيض لذات الحشرة مختلف عن الخلايا المرجعية و التى تراوحت الأعداد فيها بين ٦٨-٢٦٣ كروموسوم فى طورها الميتافيزى.