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GROWTH STUDIES OF PANLEUKOPENIA VIRUS ON TISSUE CULTURE

(With 3 Tables, One Figure and 4 Photos)

By

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بعض الدراسات عن فيروس الليكوبنيا الذى يصيب القطط وسلوكه فى الزرع النسيجى

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تم تمرير فيروس الليكوبنيا عترة (CU3) فى كل من خلايا ($NLFK_{234}$,$NLFK_{234}$, CRFK, A_{72}, MDCK, Vero, BHK_{21}) حيث وجد أن أنسب نوع لنمو وتمرير الفيروس هو $NLFK_{224}$. وبدراسة منحنى النمو للفيروس فى هذه الخلايا تبين أن أعلى معيار للفيروس المتعلق بالخلايا $10^{5.1}$ جرعه نصف معديه للمزارع النسيجيه /مل بعد ٧٢ ساعه من الحقن وأعلى معيار للفيروس الحر هو $10^{5.2}$ /ملى بعد ٩٦ ساعه وأعلى معيار كلى $10^{5.8}$ /ملى بعد ٩٦ ساعه. وباستخدام اختبار التلزن الدموى وجد أن أعلى معيار للفيروس المتعلق بالخلايا هو ٠,٢٥/٦٤ و٠ ملى بعد ٧٢ ساعه من الحقن وأعلى معيار للفيروس الحر هو ٠,٢٥؟١٢٨ و٠ بعد ٩٦ ساعه وأعلى معيار كلى ٠,٢٥/٢٥٦ و٠ بعد ٩٦ ساعه. وبدراسة السلوى النموى للفيروس فى الخلايا وجد أن تأثيره المرضى على الخلايا يتميز بوجود أجسام احتوائيه داخل السيتوبلازم بعد ٢٤ ساعه وفى النواه بعد ٧٢ ساعه من الحقن وقد تأكدت هذه الملاحظات باختبار الوميض الفلوروسينتى المباشر.

SUMMARY

Feline panleukopenia virus was propagated in different types of cell cultures from different origins and passages: $NLFK_{224}$, $NLFK_{319}$, CRFK, A_{72}, MDCK, Vero and BHK_{21}. It was found that the most suitable cells for virus propagation were $NLFK_{224}$. The growth curve of this cell culture showed that the highest infectivity titre for cell free virus (CFV) was $10^{5.1}$

$TCID_{50}$/ml (Tissue culture infective dose) after 72 hours post inoculation (PI), cell associated virus (CAV) $10^{5.2}$ $TCID_{50}$/ml 96 hours PI and total virus (TV) yield $10^{5.8}$ $TCID_{50}$/ml 96 hours PI. The haemagglutination titre of CAV was 64/0.025ml 72 hours PI while that of CFV was 128/0.025ml 96 hours PI and that of TV reached 256/0.025ml 96 hours PI. The cytopathic effect of the virus was characterized by the appearance of intracytoplasmic inclusion bodies after 24 hours PI and intranuclear inclusion bodies after 72 hours PI. These results were confirmed by direct fluorescent antibody technique.

Key Words: *Panleukopenia Virus-Tissue Culture-Growth.*

INTRODUCTION

Feline panleukopenia (FPL), infectious feline enteritis or cat distemper, is a highly contagious viral disease of cats which causes a high mortality in susceptible populations. It is considered to be the most important disease of cats (Scott et al., 1970). All members of the cat family (felidae) are affected as well as raccon, mink and possibly the ferret (Greene, 1990). Cat of any age can be striken, but there is a predilection for cats under 2 years of age. The disease is characterized by high fever, anorexia, vomiting, depression and leukopenia. Death may occur in 1 to 5 days after onset of clinical signs in 30 to 90% of cases. Until the mid 1960's, all experimental work with FPL had been limited. In 1964, Johnson succeeded in isolating a virus from a leopard with a disease identical to FPL (Johnson, 1965). Feline panleukopenia virus (FPLV) is classified as a member of the family Parvoviridae genus Parvovirus (Siegl et al., 1985). FPLV is not always easily diagnosed. Isolation of this virus from natural cases of FPL is obviously a more difficult task than with the majority of known viruses where infected cat tissue may contain recognizable virus concentration as low as $10^{1.5}$ $TCID_{50}$/gm (Johnson, 1965). In Egypt, there were no available data that deal with this virus or disease. Allover the world, control of this disease is by using the vaccine whatever living or inactivated tissue culture or use mink enteritis vaccine. The present investigation is designated to find the optimizing growth and culture conditions for a standard FPLV as the first step for the production of potent vaccine.

MATERIAL and METHODS

1. Virus :

FPLV strain (CU3) was kindly supplied by Dr. Parrish from James Baker Institute for Animal Health Cornell University NY, USA.

2. Cell culture and media :

Different types of cell line; Norden Laboratory feline kidney "NLFK" with two different passages. Crandel feline kidney cell "CRFK", Madin Darby Canine kidney cell "MDCK" and canine kidney cell A72. These cell lines were obtained from Cornell University, USA. Baby hamster kidney cell line (BHK21) and African green monkey cell line (VERO) were obtained from Serum and Vaccine Research Institute, Abbasia. All cell lines were propagated using Eagle's basal medium supplemented with 10% newborn calf serum for growth and 5% for maintenance with antibiotic added to a final concentration of 100ug streptomycin sulphate and IU of penicillin G sodium per ml of medium.

3. Virus propagation and production:

Virus propagation and production was carried out according to Johnson (1971). The infectivity titre was calculated according to Reed and Muench (1938).

4. Haemagglutination test (HA):

It was done according to Johnson (1971) using 1% chilled porcine red blood corpuscles at a pH of 6.8.

5. Direct fluorescent antibody technique (FAT):

The FAT was carried out according to Johnson (1971) using feline panleukopenia antiserum conjugated with fluorescein isothiocyanate (Lot 8803) which was kindly supplied by Diagnostic Lab University of Minnesota, USA. It is used for detection of FPLV antigen in infected cultures.

6. Susceptibility of different cell lines to FPLV:

The virus propagated in cell culture and cells were examined daily for the appearance of the cytopathic effect (CPE).

7. Growth patterns of FPLV:

It was carried out on NLFK cells where the cell free virus (CFV) cell associated virus (CAV) and total virus yield (TV) were titrated for infectivity and HA at regular intervals till the complete end of CPE. The

growth pattern included also examination of the tissue culture coverslips stained with Hematoxylin and Eosin (H and E) stain and FAT on the same intervals (according to Attyat, 1994).

RESULTS

1. Susceptibility of different cell lines to FPLV vaccinal strain:

From the data given in table (1), it is shown that $NLFKp_{224}$ is the most susceptible cell with obvious response associated with very intensive and sever CPE with HA activity 256/0.025ml and infectivity titre $10^{5.8}$ $TCID_{50}$/ml. The same table documents that $NLFKp_{319}$, CRFK, BHK_{21} and VERO cell line gave lower titres, while canine kidney cells A72 and MDCK, did not exhibit any response concerning CPE or HA activity.

2. Growth curve of the FPLV vaccinal strain:

Table (2) shows the sequential development of haemagglutination in relation to FPLV growth in cell culture, where there was no any detectable level of HA activity in all aliquots of virus (CFV, CAV, TV) till 12 hours post inoculation (PI). HA titre of CFV started after 24 hours and reached its maximum 96 hours PI. CAV started at 18 hours PI with low titre and reached its maximum 72 hours PI. TV started at 18 hours PI and reached its maximum 96 hours PI.

In table (3) and Fig. (1), there is a gradual increase in infectivity titre of TV harvests of FPL till it reached its peak ($10^{5.8}$ $TCID_{50}$/ml) at 96 hours PI. In case of CAV portion, the virus increased gradually till it reached $10^{5.1}$ $TCID_{50}$/ml at 72 hours PI, then slight decrease at 96 hours and eventually sharply decrease at 120 hours PI with $10^{2.9}$ $TCID_{50}$/ml.

The microscopic examination of cells stained with (H and E) revealed no noticeable changes could be detected during the 1st 24 hours, then the cells began to be rounded, darkened with granulation of the cytoplasm. The cells became detached leaving irregular spaces. These changes increased till the 4th day where most of the sheet-get detached. The remaining cell were either gathered in clumps or elongated. The intranuclear inclusion bodies appeared in the cells after 24 hours PI where wispy aggregation on mid way between the nucleolus and the nuclear membrane. This material progressively condensed to form a distinct basophilic band partially or completely encircling the nucleolus around which remained a pale halo (Photo 1 and 2).

Sequential detection of FPLV antigen in infected cells:

As shown from Photo (3), intracytoplasmic fluorescence was detected in many cells as early as 24 hours PI. This specific reaction became more intensive and diffuse at 48 hours PI. Intranuclear fluorescence became more clear and diffuse with clear CPE and appearance of perinuclear fluorescence 72 hours PI. The fluorescence reaction was developed to become more intensive at 96 hours PI but no fluorescence was shown in non-infected cells (Photo 4).

DISCUSSION

FPL is a highly contagious disease affecting family felidae. The disease is always diagnosed by animals clinic and hospitals. So, the owners of cats immunize their animals by using specific imported vaccine. Such vaccine sometimes is deprived in the markets, moreover the major aim of Veterinary Serum and Vaccine Research Institute (VSVRI) is the production of all animals and poultry vaccines. So, the workers in (VSVRI) are trying to produce a local FPL vaccine in sufficient quantities instead of the imported one. This study is a step to produce a potent living attenuated FPL tissue culture vaccine.

The 1st experiment was conducted to study the effect of cell culture type and passage on FPLV multiplication. In case of NLFK cells, it was found that the virus attained a good titre for both infectivity and HA in low passaged cell (P224) but gave lower titre at (p319). This may be due to the possibility that these cells could have lost their receptors to the virus by high serial passage. Johnson (1965) mentioned that detection of CPE of FPLV was transient, it varied in extent depending upon passage level of cells and in different batches of kidney cells. Durham et al. (1985) found that the highest yield of bovine parvovirus were obtained by the use of selected cell strain at low passage levels. Parrish (1991) (unpublished data) found that response of A72 cell line to canine parvo virus was decreased after the cell reached 180 passages. In spite of CRFK is a feline cell, the susceptibility of the cell to FPLV is considered low. Scott et al. (1970) found that FPLV produced CPE and low virus titre in five feline cell culture including CRFK. Black et al. (1979) failed to produce any cell destruction in CRFK infected with FPLV but the virus was detected only through indirect immunofluorescence. In case of canine cell origin, the virus failed to produce any CPE or HA in both A72 and MDCK. This result is similar to that obtained by Black et al. (1979), Goto et al. (1986). Tryun and Parrish

(1992) mentioned that FPLV replicated only in feline cells, while Veijalainen (1988) found very slight replication in the 2 types of cells. Mochizuki et al. (1986) mentioned that growth of FPLV (TU1 strain) in certain MDCK was obtained by serial subculture, but, the strain passaged 10 times did not gain the ability to infect other canine cells. The FPLV propagated in BHK_{21} gave a titre of $10^{3.5}$ $TCID_{50}$/ml after the 6th passage. So, the virus may need more propagation to increase the titre.

In case of Vero cells, the CPE began after the 8th passage. Eugster (1980) and Pardiso et al. (1982) obtained also a low titre for canine parvo virus $10^{2.5}$ $TCID_{50}$ in this cell.

In this study, the growth curve of FPLV on NLFK (Tables 2 and 3) showed that both CFV and CAV were increasing in parallel lines for infectivity and HA. These results are confirmed by Hirasawa et al. (1985) who found in synchronized CRFK both infectivity and HA titres began to rise in the cellular phase 12 hours PI and in the extracellular 24 hours PI, then reached its maximum at 48 and 72 hours, respectively. Also, Zuffa (1987) found that FPLV increased both intra and extracellular until the 3rd day, then the extracellular particles levelled off and decreased after the 4th day, while the intracellular virus peaked on the 6th day.

From table (3), the assay of FPLV by HA was only possible after virus infectivity reached $10^{2.5}$ $TCID_{50}$/ml but not before that where a very low HA titre was obtained. Similar findings were reported by Johnson (1971) and Konishi et al. (1975) who could not detect HA activity of FPLV in culture unless the virus infectivity reached more than 10^5 $TCID_{50}$/ml. Wosu (1987) obtained the same results with FPLV and porcine parvovirus with a very low HA titre ranging from 2-8, but he observed a gradual increase in HA titre from the 2nd PI to reach its maximum (128) at the 5th day PI; this is in consistency with our obtained data.

The microscopic detection of the developing CPE in the present work was also obtained by Johnson (1965), and Scott et al. (1970). The use of the more sensitive FAT described the sequential development of the fluorescence reaction in both cytoplasm and the nucleus of infected cells. The intranuclear positive fluorescence has been documented by Johnson (1965) and Black et al. (1979). The work done by Zajac et al. (1988) supports our observation concerning the development of both intracytoplasmic and intranuclear fluorescein infected cell as early as 24 hours PI.

From the results of the present investigation it can be concluded that the optimal conditions to get the best FPL, in order to prepare a potent

vaccine is to grow the virus on $NLFK_{224}$ cell culture and harvesting the whole virus fractions 96 hours PI.

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Table (1) Susceptibility of different cell culture to FPLV vaccinal strain.

Type of Cell line	Time of appearance of CPE	Degree of CPE (7 days PI)	HA titre/0.025ml	Infectivity Titre $TCID_{50}/log_{10}ml$
$NLFK_{P224}$	1st passage	++++	256	5.8
$NLFK_{P319}$	3rd passage	++	4	2.5
CRFK	4th passage	+	8	2.8
A72	-	-	0	Not done
$MDCK_{259}$	-	-	0	Not done
Vero	8th passage	++	8	3.2
BHK_{21}	6th passage	++	16	3.5

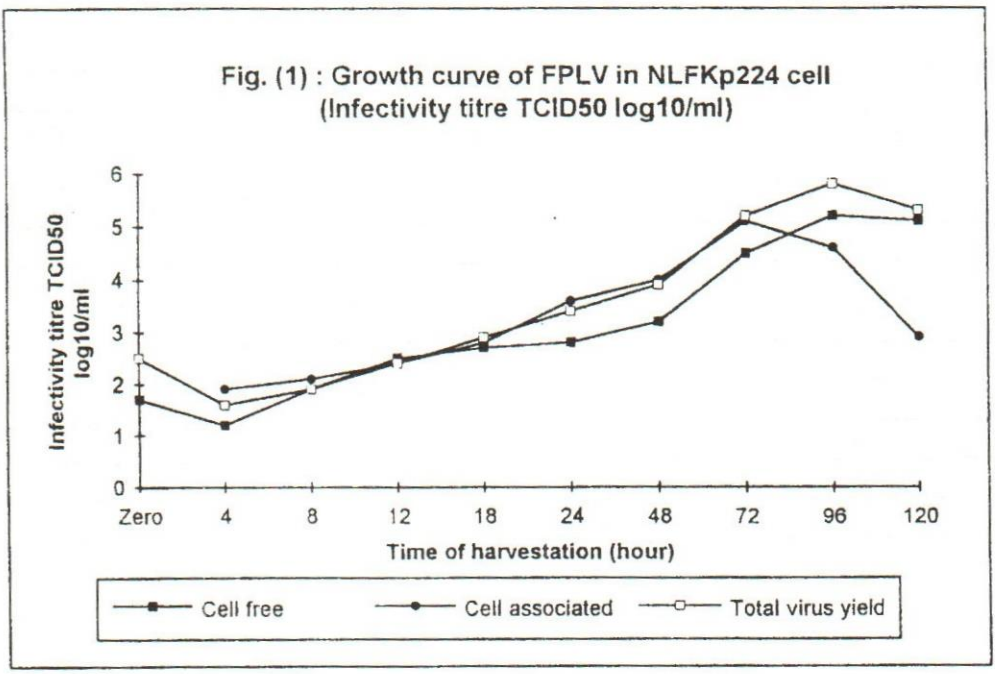
CPE : Cytopathic Effect. HA : Haemagglutination test.
PI : Post Inoculation.
$TCID_{50}$: Tissue Culture Infective Dose.
- : Indicates no response.

Table (2) : Haemagglutination titre of FPLV vaccinal strain in relation to virus growth on $NLFK_{p224}$ cell culture.

Time of harvestation per hour	Haemagglutination titre / 0.025ml		
	Cell free virus	Cell associated virus	Total virus yield
Zero	0	0	0
4	0	0	0
8	0	0	0
12	0	0	0
18	0	2	4
24	2	8	8
48	8	32	16
72	32	64	64
96	128	64	256
120	128	16	256

Table (3) : Growth curve of FPLV in $NLFK_{p224}$ cell assayed by infectivity titration.

Time of harvestation Per hour	Infectivity titres $TCID_{50}$ log_{10}/ml		
	Cell free virus	Cell associated virus	Total virus yield
Zero	1.7	Not done	2.5
4	1.2	1.9	1.6
8	1.9	2.1	1.9
12	2.5	2.4	2.4
18	2.7	2.8	2.9
24	2.8	3.6	3.4
48	3.2	4.0	3.9
72	4.5	5.1	5.2
96	5.2	4.6	5.8
120	5.1	2.9	5.3



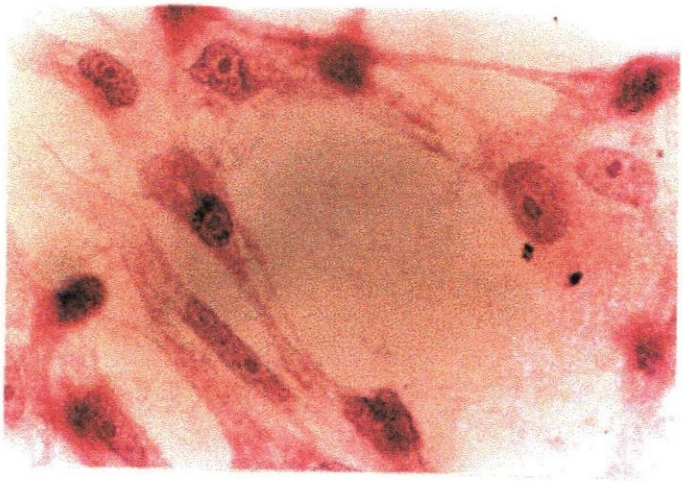


Photo (1) : Infected NLFK cells with FPLV showing intranuclear inclusion bodies and elongation of the cells (1000X) (H & E).

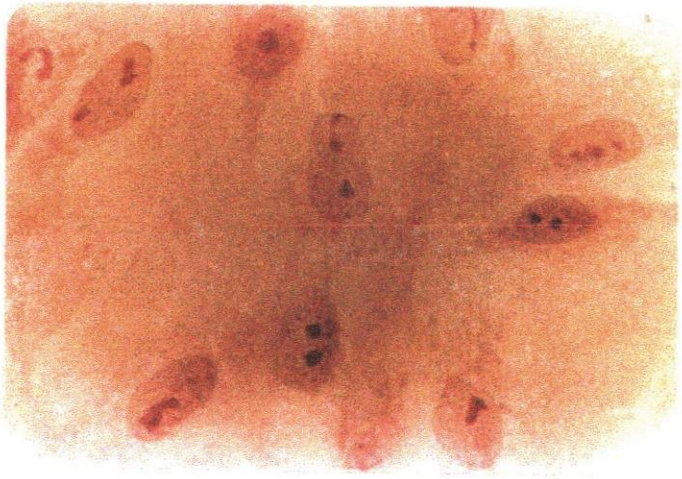


Photo (2) : Normal uninfected NLFK cells (H & E).

Photo (3) : Infected NLFK cells with FPLV (CU3) showing intra- and perinuclear fluorescence with clear CPE (400X).

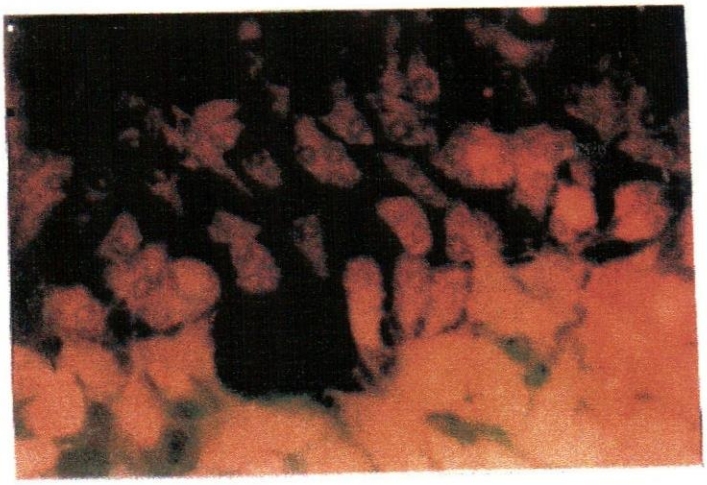


Photo (4) : Normal uninfected NLFK cells (400X).