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Adaptation of locally isolated equine herpesvirus (EHV-1) on different cell lines

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Locally isolated EHV-1 (2002) was successfully adapted to different available cell lines (BHK, vero, MDBK and MDCK). Characteristic cytopathic effect (CPE) was observed from the first passage and the virus growth became regular with constant incubation periods at 2nd day in BHK and vero cells 4th day in both MDBK and MDCK cells post inoculation with maximum titers of 7, 8.5, 7.75, 6.2 log_{10} $TCID_{50}$/ml in BHK6, vero10, MDBK5 and MDCK5 cell lines; respectively. Growth kinetics of EHV-1 in vero cells, showed initial increase in cell associated viruses (CAV) and cell free viruses (CFV) were at 8 and 48 h. post inoculation then reaching their peak at 72 and 96 h. post inoculation in CAV and CFV; respectively. Detection of viral protein in infected cells was done using IFT. It revealed that high fluorescence was shown at 4 till 72 h. post inoculation in CAV and 72 h. in CFV. In conclusion, EHV-1 is more cell associated and IFT is considered as a rapid and sensitive test for detection of viral protein

EHV-1 is the major cause of abortion at the last trimester of pregnancy in equines (Allen and Bryans, 1986, O'Callaghan ; Osterrieder, 1999). Moreover, it causes a respiratory manifestation in newly born foals and yearling, and nervous syndrome in adults. EHV-1 was propagated in equine kidney cells with formation of intranuclear inclusions and CPE after 2-5 days (Shimizu *et al.,* 1957). Also, it could be grown in rabbit and ovine kidneys (Plummer and Waterson, 1963; Girard *et al.,* 1963), liver of chicken embryo and kidney epithelium of G. pig (Zhelev and Semerdzhiev, 1962; Randall and Lawson (1962) also they reported that EHV-1 may be proliferated in Hella cells then passaged in L cells. Mahnel and Hartl (1971); Kukriea *et al.,* (1998) stated that the antigens of EHV-1 propagated in BHK cells were superior to that prepared from primary kidney cells. EHV-1 was extensively passaged in bovine kidney (Sugahara *et al.,* 1994) and can be isolated in Madin Darby bovine kidney (MDBK) cells (OIE, 2000). Also, EHV-1 could be propagated in rabbit kidney (RK-13) (De-Simon and Lodetti, 1971; Dutta *et al.,* 1983) and in vero cells (O'Callaghan and Osterrieder, 1999; OIE, 2000).

Rattan *et al.,* (1999) adapted EHV-1 in donkey kidney cells (DKC) producing a characteristic CPE. The virus titres were equivalent to those obtained from horse kidney cell culture. The present study was planned to propagate EHV-1 in different cell lines (vero, BHK, MDBK and MDCK) and a comparison occurred between them and egg propagated virus. The produced virus could be used as antigen in serological tests as well as in vaccine preparation.

Material and methods

Virus. Locally isolated Egyptian strain of EHV-1 isolated from aborted mares (Hassanein *et al.,* 2002; Safaa, 2003). The estimated virus infectivity titre was 6.3 $TCID_{50}$.

Cell cultures.

African green monkey kidney cells (vero). It was obtained from FADDL, Plum Island, USA.

Madin Darby canine kidney cells (MDCK). It was supplied by VACSERA, Wl-Agouza, Giza, Egypt.

Madin Darby bovine kidney cells (MDBK). It was supplied by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Dept. of Rinderpest Like Diseases.

Baby hamster kidney cells (BHK). Both MDBK and BHK were obtained from Rift Valley Fever Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. These cell lines were used for virus propagation and adaptation.

Embryonated chicken eggs (ECE). Specific pathogen free (SPF), 11-13 days, were used for virus isolation and propagation. It was obtained from SPF Farm, Koum Osheim, Fayoum, Egypt.

Biological materials.

Rabbit anti-EHV-1 hyperimmune serum conjugated with FITC. It was locally prepared according to (Hudson and Hay, 1989) in Equine Diseases Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Standard lapinized antisera against EHV-1 and EHV-4. They were kindly supplied by Dr. Jannet Wellington, Dept. of Biological Science, Macquarie Univ., MSW, Australia. It was used for virus identification.

Virus propagation and adaptation on cell lines. It was applied according to (Dutta *et al.,* 1983). Different cell cultures were grown with Eagle's minimal essential medium (EMEM) which had been supplemented with 10% newly born calf serum (NCS, and antibiotics, penicillin, 100 U/ml; streptomycin, 100 µg/ml). Monolayer cultures was washed 3 times with phosphate buffer saline (PBS) pH 7.2 to remove the remained serum, then it was inoculated with 0.5 ml stock virus with infectivity titre 6.2 $\log_{10}$ $TCID_{50}$/ml. After incubation at 37°C for 1 hour, washed twice with PBS then EMEM with antibiotics but without NCS was added. The 4 onze bottles were incubated at 37°C and examined daily, the virus was harvested when maximum cytopathic effect was observed and it was serially passaged. Virus infectivity titre was assayed according to (Reed and Muench, 1938).

Virus propagation on ECE. I was applied according to (Warda, 2003). Locally isolated virus was inoculated into chorioallantoic membrane.

Virus identity and purification. Virus purification was done according to (Azmi and Field 1993) and identity was made by SNT using reference antisera against EHV-1 and EHV-4 (Bernhardt, 1993).

Growth curve of the 4th passage of EHV-1 on vero cells. Aliquots of tissue culture fluid were harvested at different intervals of time 4, 6, 24, 36, 40 and 96 h. post infection (PI). Virus titration of both cell free virus (CFV) and cell associated virus (CAV) were assayed (El-Kabbany, 1994).

Detection of EHV-1 by direct IFT. Studying the development of specific viral protein of both CFV and CAV (Khaled *et al.,* 2004) using the locally prepared anti-EHV-1 serum conjugated with FITC (Warda *et al.,* 2006).

Results and Discussion

EHV-1 is contagious disease affect all ages of equines, causing abortion in pregnant mares, perinatal mortality and respiratory manifestation in yearling and neurological syndrome in adults. So, vaccination should be recommended.

The potency of the tissue culture vaccine is mainly related to the antigenicity of the harvested virus and its infectivity titre which showed variations within different cell lines.

The aim of this work is based on detection of the best line which is valid for EHV-1 isolation and propagation as well as for vaccine production. So, BHK, vero, MDBK and MDCK cell lines were used.

Table (1) showed that CPE in BHK cells was detected in the first passage at 4 days post inoculation till it reached 2 days at the 6^{th} passage while CPE in MDBK cells was detected at 7 days till reaching 4 days at 5^{th} passage meanwhile, in MDCK it was 4 days at the 5^{th} passage.

CPE was developed as rounding and ballooning with some syncytial formation. These changes became generalized and destroyed the cell sheet. This finding coincidence with that found by Plummer and Waterson (1963); Shimizu *et al.,* (1957); OIE (2000).

Also table (1), showed the infectivity titre of different infected cell lines revealed that the maximum titre of EHV-1 adapted on BHK, vero, MDBK and MDCK were 7, 8.5, 7.75 and 6.2 $\log_{10}$ $TCID_{50}$/ml, respectively.

vero cell line was used as a model to study the growth kinetic of EHV-1 due to its high titre at the same passage compared with the other used cell lines.

The growth kinetics of EHV-1 on vero cells (table 2) showed initial increase in the titre of CAV and decreased CFV occurred between 8 and 48 h. post inoculation (hrs PI) then continued to increase reaching their peak at 48 and 120 hrs PI respectively.

Concerning to IFT (Table 3, Photos 1, 2), it revealed that high nuclear fluorescence was shown starting at 4 till 72 hrs PI in CAV while in CFV was at 72 and 96 hrs PI.

This result coincidence with Darlington and James (1966); Weiland and Dini-Troides (1970); Khaled *et al.,* (2004) who observed the first fluorescence in the infected cells appeared in the nucleus at 4 hrs PI as a homogenous mass of fluorescent material compared with negative control. Also, it was present in the perinuclear cytoplasm at 6 hrs PI then the viral antigen accumulated at the cell surface. IFT in this work showed its great sensitivity and validity for detection of CAV moreover, it could be detected earlier before the development of CPE (Gunn, 1992; OIE, 2000).

The infectivity titre of egg adapted virus was 9.5 EID_{50}/0.2 ml. However, cell culture propagated virus is more easy, soft handling and sterile.

From aforementioned data, the conclusion was:

Table (1): Infectivity titre of EHV-1 in different cell lines.

No. of passage on different cell lines	Incubation period	Infectivity titre (log_{10} $TCID_{50}$/ml)
I. BHK cells		
1st passage	4 days	6.20
4th passage	3 days	6.75
6th passage	2 days	7.00
8th passage	2 days	7.00
II. vero cells		
1st passage	5 days	7.50
3rd passage	5 days	8.00
8th passage	2 days	7.75
10th passage	2 days	8.50
11th passage	2 days	8.50
III. MDBK cells		
1st passage	7 days	6.25
3rd passage	6 days	6.25
4th passage	5 days	6.75
5th passage	4 days	7.75
8th passage	4 days	7.75
IV. MDCK cells		
1st passage	5 days	6.00
3rd passage	4 days	6.25
5th passage	4 days	6.25

Table (2): Growth kinetic of infected vero cells with the 3rd passage of locally isolated EHV-1.

Time post inoculation (hours)	Infectivity titre (log_{10} $TCID_{50}$/ml)	
	Cell associated virus	Cell free virus
4	4.0	6.55
8	4.2	ND
24	5.5	4.0
36	5.8	4.0
48	6.3	5.0
72	6.3	6.3
96	6.0	7.0
120	6.0	8.0

Table (3): Correlation studies between CPE and IFT of infected vero cells with EHV-1.

Time post inoculation (hours)	IFT		CPE
	Cell associated virus	Cell free virus	
4	+++	++	-
8	+++	+	-
24	+++	+	-
36	+++	++	+
48	+++	++	+
72	++	++	+++
96	+	++	+++

CPE: Cytopathic Effect
IFT: Immunofluorescent technique

vero, BHK, MDBK and MDCK cell lines could be used for propagation and adaptation of EHV-1. EHV-1 is more cell-associated (Darlington and James 1966; Weiland; Dini-Troides, 1970). IFT is considered as rapid and sensitive test for detection of viral protein in these infected cells.

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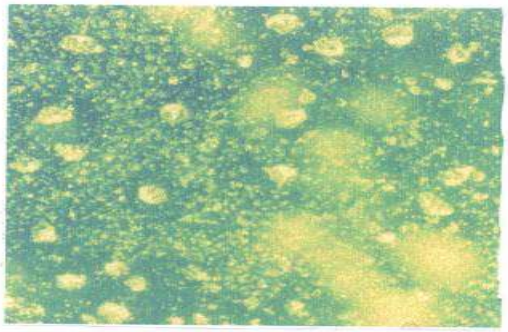

Photo (1): Diffuse and intensive cytoplasmic fluorescence of infected VERO cells with EHV-1 stained by FAT (Mag. 40x)

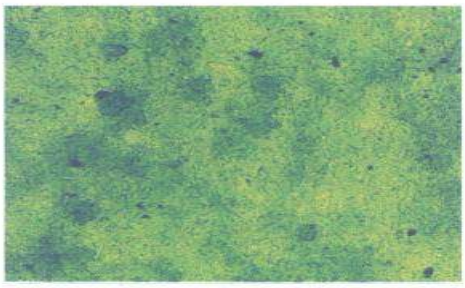

Photo (2): Normal sheet of non-infected VERO cells, with faint greenish illumination (Mag. 40x)

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تمرير وأقلمة فيروس الإجهاض المعدى المعزول محلياً من الخيول على خلايا نسيجية مختلفة

تم بنجاح أقلمة فيروس الإجهاض المعدى المعزول محلياً من الخيول على أربعة أنواع من خلايا الزرع النسيجى (vero, BHK, MDBK, MDCK). ولقد وجد أن معدل نمو الفيروس أصبح منتظم ويعطى فترة حضانة ثابتة وهى يومين فى خلايا vero، BHK ٤ يوم فى كلا من خلايا vero, MDBK وكان أعلى معدل عيارية هى ٧، ٨,٥، ٧,٧٥، ٦,٢ جرعة نصف معدية/ملى فى خلايا BHK، vero، MDBK، MDCK على التوالى وقد قسم منحنى نمو الفيروس فى خلايا vero وكانت على عيارية للفيروس المرتبط بالخلايا والفيروس الحر هى ٤٨، ١٢٠ ساعة بعد الحقن على التوالى. أيضاً تم رصد البروتين الفيروسى بواسطة اختبار الفلورسين المناعى واظهر أعلى درجة عند ٤، ٤٨ ساعة بعد الحقن فى حالة الفيروس المرتبط بالخلايا، ٧٢ ساعة فى الفيروس الحر. قد استنتج من هذه الدراسة أن الفيروس أكثر ارتباطاً بالخلايا وكذلك اختبار المناعى المشع حساس واسرع اختبار للكشف عن البروتين للفيروس.