INACTIVATION OF VERO CELL CULTURE ADAPTED CHICKEN ANEMIA VIRUS VACCINE

Samah, S. Abou-Dalal [1], El-Bagoury, G.F [2], Suzan, K.Tolba[1], Khodeir, M.H. [1]

[1] Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.[2] Department of virology, Faculty of Veterinary Medicine, Benha University.

ABSTRACT

CAV is usually propagated on MDCC cell-culture and because this type of cells are not available in Egypt. We have used Vero cell line instead. CAV was propagated fourteen serial passages in Vero cell culture. The cytopathic effect in cell culture (CPE) was characterized vacuolation of the infected monolayers and subsequent detachment. Vacuolation of the infected monolayers started by the 7th day post infection (DPI) then began to appear more early by the successive passage to reach the 2nd DPI cell. The highest virus titer was 7.6 $\log_{10}TCID_{50}$ /ml in Vero cell culture obtained by the 10th passage. Studying the growth kinetics of CAV in Vero cell culture indicated that the highest total virus yield was 7.6log10TCID50/ml by 72 hours post cell infection. Complete inactivation of CAV was obtained after 72 hours at 37oC using 0.2% formalin. Complete virus inactivation was confirmed by 3 successive passages in Vero cell culture showing absence of CPE and FAT showing negative reaction.

Keywords: CAV, Vero, Inactivation, Formalin

(BVMJ-25(2):55-62, 2013)

1. INTRODUCTION

Chicken anemia virus (CAV) is a ubiquitous and highly resistant virus of chickens that causes anemia and death in chicks less than 3 weeks of age and immunosuppressant in chickens older than 3 weeks of age [1].

The isolation of virus in SPF chicks or in cell cultures (MDCC-MSBI cells) is laborious and time consuming so that, the virus infection has been diagnosed rapidly by detecting the virus DNA by PCR in tissue samples from infected birds [2]. Susceptibility of MDCC to CAV was measured by the number of cells positive for viral antigen in immunofluorescence (IF) tests at 3-10 days post infection using direct florescent antibody technique [3].

In Egypt, a local isolate of CAV was successfully propagated in VERO and CEF cells where the CPE was characterized by vacuolation of the infected monolayers and subsequent cell detachment while no obvious CPE was detected in BHK and MDBK cell cultures [4], [5]. For CAV inactivation, 0.2% formalin was used for 72 hours at 37oC [6], [7].

The present work aims to inactivate CAV adapted on Vero cell culture as a preliminary study to prepare an inactivated vaccine.

2. MATERIALS AND METHODS

2.1. Virus strain:

Commercial chicken anemia virus (CAV) vaccine adapted and propagated on MDCC cell line was kindly supplied by Inter Vet Company. CAV-VAC is live virus vaccine

prepared from a modified US field isolate of chicken anemia virus (CAV).

2.2. African green monkey kidney cell line (VERO).

It was kindly supplied by the Department of Pet Animal Vaccine Research; Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

2.3. Growth kinetics of CAV in cell cultures.

The used cell cultures were seeded in Leighton's tube containing cover slips then infected with the highest virus passage where the cell free; cell associated and total virus yield were determined on regular intervals post cell infection till harvesting.

2.4. Staining of cell cultures:

Normal and infected cell cultures prepared on cover slips were stained with hematoxilin and eosin to demonstrate the induced CPE (8).

2.5. Virus titeration:

Titrations of the propagated CAV in Vero cell culture was carried out using the micro-titer technique [9] and the virus titer was calculated as log10TCID50/ml [10].

2.6. *CAV hyper immune serum conjugated with fluorescent isothiocyanate:*

CAV hyper immune serum, prepared in chickens and conjugated with fluorescent isothiocyanate was supplied kindly by VSVRI and used in the direct FAT to confirm the presence of CAV in the used infected cell.

2.7. Direct fluorescent antibody technique (FAT).

Direct FAT was carried out on infected cell cultures using conjugate of hyper immune serum against CAV [9].

2.8. Virus inactivation.

The CAV pool obtained from Vero cell culture was freeze-thawed three times, and centrifuged at 15000 rpm, for 20 min, to eliminate the cell debris. The CAV suspension was inactivated by the addition of 0.2% formalin solution and incubation at 37°C for 72 h.

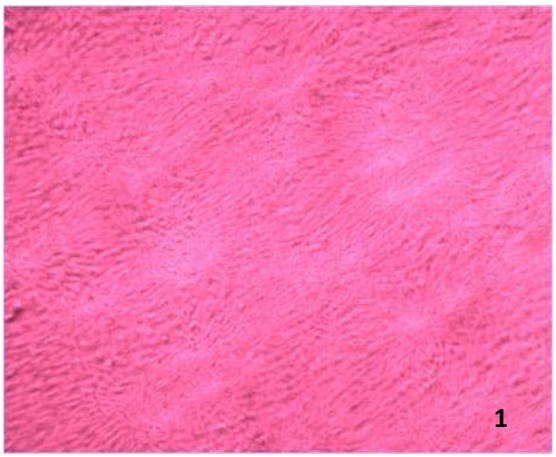
The complete inactivation of the CAV virus was checked by making 3 serial passages in Vero cell culture, and checking the absence of virus by direct immunofluorescence [6].

Samples were obtained from the virus formalin mixture every 6 hours in screw capped tube and tested by virus titration to ensure complete virus inactivation. Inactivation process was stopped by addition of cold sodium thiosulphate with a final concentration of 2% and the inactivated virus was transferred into another clean container after inactivation to ensure that no virus fluid escaped during inactivation process on the neck of the vessel.

3. RESULTS

Propagation of CAV in African green monkey kidney (Vero), showed that the cytopathic effect (CPE) was characterized by vacculation of the infected monolayers and subsequent cell detachment.This result was demonstrated in photo (2) in comparison to normal control Vero cells in photo (1).

Photo 1. Normal Vero cell culture (H&E-40x)



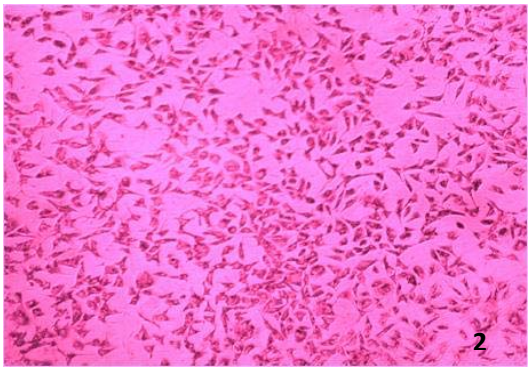


Photo 2. Vero cell culture infected with CAV (H&E-40x) showing cell detachment and subsequent vacculation

Vacculation of the infected monolayers started by the 8th day post infection (DPI) then began to appear more early by the successive passage to reach the 2nd DPI.

Examination of infected Vero cell culture using direct fluorescent antibody technique (FAT) with specific antiserum conjugated with fluorecin-isothyocyanate confirmed that the detected CPE in infected Vero cell culture is induced by the CAV. The technique revealed the presence of intra nuclear inclusions reflected by positive FAT as apple green reaction in comparison to normal control Vero cells showing absence of specific fluorescence.

Serial propagation of CAV in African green monkey kidney (Vero), showed that the virus titer is increased gradually by serial passage and the highest virus titer was 7.6 log10TCID50/ml obtained by the 10th virus passage. These results are tabulated in table (1).

Studying the growth kinetics of CAV showed that, in the first hours post cell culture infection, it was noticed that the cell associated virus titer was higher than that of the cell free then began to become of lower values. The highest total virus yield was obtained by 72 hours post cell infection (7.6log10TCID50/ml) as demonstrated in table (2).

Table (1): Effect of serial passage of CAV in Vero cells on onset of CPE and virus titer.

Number of Virus passage	Onset of CPE	Time of harvesting	Virus titer ($log_{10}TCID_{50}$/ml)
1	8DPI*	10DPI	0.5
2	8DPI	9DPI	0.8
3	7DPI	9DPI	1.5
4	6DPI	8DPI	2.5
5	5DPI	7DPI	4.0
6	4DPI	6DPI	5.0
7	3DPI	5DPI	6.0
8	2DPI	3DPI	7.0
9	2DPI	3DPI	7.6
10	2DPI	3DPI	7.6

*DPI= days post infection

It was found that Vero cell culture adapted CAV, harvested at its 10th passage and subjected to inactivation process using 0.2% formalin for 72 hours at 37oC, lost its infective titer in a liner manner where it was 0.5 log10TCID50/ml/6hours from 6-48 hours then the virus loss was 1.0 log10TCID50/ml/6hours from 54-72 hours reaching complete inactivation by 72 hours post starting of the inactivation process as demonstrated in table (3) and figure (3). Complete virus inactivation was confirmed by 3 successive passages in Vero cell culture showing no detection of CPE and FAT showing negative reaction.

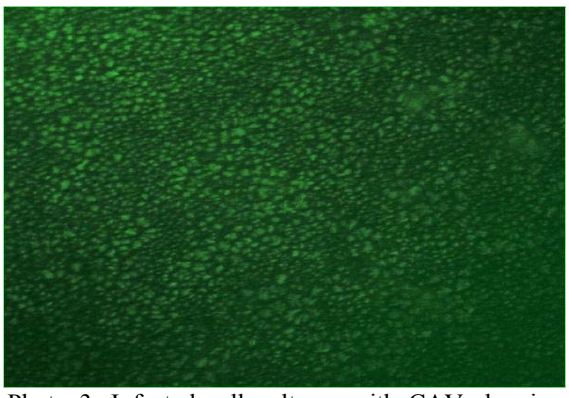


Photo 3. Infected cell cultures with CAV showing positive FAT as apple green reaction (40 xs)

Table (2): Growth kinetics of CAV in VERO cell culture

Hours post cell infection	Virus Titer (log_{10} $TCID_{50}$/ml)		
	Cell Free Virus	Cell Associated Virus	Total Virus Yield
1	0	≤ 0.5	≤ 0.5
2	0	0.5	0.6
3	≤ 0.5	1.0	1.5
4	0.6	2.0	2.1
5	0.8	2.4	2.5
6	1.0	4.0	3.8
12	1.5	4.5	5.0
36	5.0	2.0	6.5
48	5.7	1.0	7.0
60	6.5	1.0	7.0
72	7.0	0.5	7.6

Table (3): Loss of CAV titer through the inactivation process

Hours of inactivation	Virus titer ($log_{10}TCID_{50}$/ml)	Loss in virus titer ($log_{10}TCID_{50}$/ml)
0	7.6	0.0
6	7.5	0.1
12	6.5	↑
18	6.0	
24	5.5	
30	5.0	0.5
36	4.5	
42	4.0	
48	3.5	↓
54	2.5	↑
60	1.5	1.0
66	0.5	↓
72	0.0	

4. DISCUSSION

In this respect there were very little available data that described propagation of CAV in Vero cell culture but it was reported that chicken anemia agent (CAA) can be propagated in an established cell line derived from Marek's disease (MD) lymphoma (MDCC-MSB1)(11). The cytopathic effect of CAV in the infected MSB-1 cells was first evident following the fifth passage [12]. In vitro conditions, CAV replicates only in transformed chicken cell lines, which indicate that at least a part of the CAV life-cycle requires transformed-like cellular events [13]. In these transformed cell lines, the synthesis of the apoptin protein alone mimics the CAV-induced apoptosis, whereas the VP2 protein also harbors some apoptotic activity. Extensive studies on apoptin resulted in the characterization of domains essential for its apoptotic activity and nuclear localization, which seems to be related with its ability to induce apoptosis. Therefore, both VP2 and apoptin are of interest in reducing the pathogenicity of CAV infections. A series of biomedical studies on apoptin have been carried out in human cell systems, which are informative about the mechanism of CAV-induced apoptosis in chicken (transformed) cells. Synthesis of apoptin alone induces apoptosis in various human transformed and/or tumorigenic cell lines, but not in normal human diploid cells.

CAV propagatedin Vero cells showed a CPE that characterized by vacuolation of the infected monolayers with subsequent cell detachment. Vacuolation of the infected monolayers started by the 8th DPI then began to appear more early by the successive passages to reach the 2nd DPI. These results come in agreement with the works in which CAV is propagated in CEF and Vero cell cultures [4], [5], [14], [15].

To confirm that the detected CPE in infected Vero cell culture is induced by the CAV; direct fluorescent antibody technique (FAT) was carried out on infected cell cultures using specific antiserum conjugated with fluorecin isothyocyanate. The technique revealed the presence of intra nuclear inclusions reflected by positive FAT as apple green reaction.

FAT was used for detection of CAV agent in infected cells by many workers, the incidence of CAV in infected CEF and Vero cell culture was confirmed through the application of direct FAT [5]. Cux1 isolate of chicken anemia virus (CAV) was passaged over 320

times in Marek's disease virus transformed chicken lymphoblastoid (MDCC-MSB1) cells and indicated that the yields of infectious virus increased with passage number and the viral antigen was detected using FAT [16]. The presence of antibodies against CAV in breeder flocks in Argentina is determined by indirect immune fluorescence testes [17]; infection of mononuclear cells derived from various chicken lymphoid tissues with CAV was detected by (IF) staining of cytospin preparations of mononuclear cells [18], which shown that the greater numbers of cells containing CAV antigens were in spleen and bone marrow cultures than in thymus cultured under the same conditions. A serological survey of antibodies against CAV was conducted in some commercial chicken flocks by indirect immune fluorescence technique (IFT) using MSB-1cells (lymphoblastoid cell line from Marek's disease lymphoma) [19].

Apoptin, a small protein encoded by chicken anemia virus (CAV) was expressed in various human hematologic malignant cell lines derived from leukemia and lymphoma by immune fluorescence staining [20].

A serological survey on the prevalence of chicken anemia virus (CAV) infection was performed by using the indirect immune fluorescence (IF) and the virus neutralization (VN) tests in commercial Hungarian chicken populations. By the indirect IF test, a total of 846 serum samples from 13 meat-type parent flocks of two breeds were investigated between 10 and 62 weeks of age [21].

Infected MSB-1 cells following at least five blind passages of chicken anemia virus was identified by using technique indirect immune fluorescence technique (IFT) [12];

Susceptibility of cell lines represented six phenotype groups of T cells based on the expression of CD4, CD8, TCR-2and 3surfases markers to CAV infection was evaluated by the number of cells positive for viral antigen in immune fluorescence test at 3-10 days post infection (3). They mentioned that cell susceptibility to CAV was measured by the number of cells positive for viral antigen in immune fluorescence (IF) tests at 3-10 days post infection.

In Egypt CAV specific antigen was demonstrated using immune-fluorescence, in infected MDCC-MSB1 cells [22] and in EAH-infected MDCC-MSB1 cells, derived from Marek's disease lymphoma, with the use of a monoclonal antibody specific for CAV [23].

CAV specific immune fluorescence (IF) and cytopathic effect were detected at very low levels at 2 days post infection, with an additional passage of infected cells into fresh medium being required to produce high levels of infectious virus [24]. Indirect fluorescent antibody technique identified infected MSB-1 cells following at least five blind passages.

Studying the growth kinetics of CAV showed that on the first hours post cell culture infection, it was noticed that the cell associated virus titer was higher than that of the cell free then began to become of lower values. The highest total virus yield was obtained by 72 hours post cell infection (7.6log10TCID50/ml). Closely similar results reported that the Cux1 isolate of chicken anemia virus (CAV) was passaged over 320 times in Marek's disease virus transformed chicken lymphoblastoid (MDCC-MSB1) cells and indicated that the yields of infectious virus increased with passage number [5], [16]. It was also found that Vero cell culture yielded the CAV titer higher than that obtained by CEF [4].

Vero cell culture adapted CAV at its 10th passage was subjected to inactivation process using 0.2% formalin for 72 hours at 37oC. It was found that the loss in virus titer was liner where it was 0.5

$log_{10}TCID_{50}$/ml/6hours from 6-48 hours then the virus loss was 1.0 $log_{10}TCID_{50}$/ml/6hours from 54-72 hours reaching complete inactivation by 72 hours post starting of the inactivation process. Complete virus inactivation was confirmed by 3 successive passages in Vero cell culture showing no detection of CPE and FAT showing negative reaction. There are no available data fully describe or discuss the inactivation of CAV but the present results come in agreement with the study which obtained complete inactivation of CAV with 0.2% formalin after 72 hours confirming such inactivation by FAT which showed absence of CAV in infected cell cultures [6] and [7].

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تثبيط فيروس أنيميا الطيور المعدي.

سماح السيد أبودلآل [1]، جبر فكرى الباجورى [2]، سوزان كامل طلبة[2] ، محمد حسن خضير [2]

[1] معهد بحوث الأمصال واللقاحات البيطرية- العباسية- القاهرة ، [2] قسم الفيرولوجيا - كلية الطب البيطرى - جامعة بنها

الملخص العربي

غالبا يتم تمرير فيروس أنيميا الطيور على خلايا MDCC ونظرا لعدم توافر هذا النوع من الخلايا فى مصر أستخدمنا خلايا خلاياكلي القرد الاخضر الافريقى بدلا منها. تم تمرير فيروس أنيميا الطيور عشر مرات متتالية فى خلايا كلي القرد الاخضر الافريقى. تم ملاحظة التأثير المرضى للفيروس علي هده الخلايا ممثلة في استدارة الخلايا ثم ظهور فجوات بين خلوية ثم موت الخلايا وانفصالها عن سطح الاستزراع. بدأظهور الفجوات البين خلوية فاليوم السابع بعد العدوي ثم ظهرت مبكرا في التمريرة التالية الي ان بدأت فى الظهورفى اليوم الثاني بعد العدوي. وقد وجد أن اعلى عيارية للفيروس فى خلايا كلي القرد الاخضر الافريقى تبلغ 7.6log10TCID50/ml عند التمريرة العاشرة. بدراسة منحني النمو للفيروسفىخلايا كلي القرد الاخضر الافريقى وجد أن أعلى عيارية كلية للفيروس كانت بعد 72 ساعة من الحقن (7.6log10TCID50/ml). تم تثبيط الفيروس تماما باستخدام مادة الفورمالين بتركيز0.2% وتمت عملية التثبيط بعد معالجة الفيروس لمدة 72 ساعة عند درجة حرارة 37 درجة سيليزيةوللتأكد من تمام عملية التثبيط تم عمل 3 تمريرات متتالية للفيروس المثبط في خلاياVero حيث لم تظهر أي أثار مرضية علي الخلايا وأعطي نتيجة سلبية لاختبار الوميض الفلوريسنتي المناعي المباشر.

(مجلة بنها للعلوم الطبية البيطرية: عدد 25(2):55-62, ديسمبر 2013)