GROWTH CHARACTERISTICS OF LOW PATHOGENIC STRAIN OF AVIAN INFLUENZA VIRUS (H5N2) IN DIFFERENT CELL CULTURES

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A B S T R A C T

A low pathogenic strain of avian influenza virus (H5N2) was successfully propagated in both primary chicken embryo fibroblast (CEF) cells and cell line of baby hamster kidney (BHK-21), African green monkey kidney (Vero) and Madine Darby canine kidney (MDCK) cells through ten successive passages; the virus induced cytopathic effect (CPE) from the 1st passage characterized by cell rounding, elongation and cell disintegration. The most suitable cell cultures for virus propagation were MDCK and BHK-21 cells that gave the highest titer 107.5 and 107 TCID50/ml respectively at the 10th passage while the highest titer for Vero and CEF cells were 105.5 and 106.5 TCID50/ml respectively at the 10th passage. H5N2 virus was cell free virus with higher titer comparing to the cell associated virus in both BHK-21 and MDCK cell line

Key Words: avian influenza virus (H5N2), CEF, BHK-21, MDCK

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1. INTRODUCTION

Influenza viruses are members of the family Orthomyxoviridae composing of 4 genera, A, B, C and Thogotovirus based on the basis of the nucleocapsid or matrix antigen [1]. However, only type A influenza viruses are able to infect and cause severe disease among variety of avian and mammalian species [2]. Influenza virus is an enveloped virus containing 8 segments of single stranded negative-sense RNA genomes. The envelope contains haemagglutinin (HA) and neuraminidase (NA) proteins. Seventeen serotypes of HA (H1~H17) and nine (N1~N10) of NA have been identified in both mammalian and avian influenza type A [3]. The viral particles are approximately 50-120 nm in diameter for spherical forms [1]. Most laboratory-adapted influenza viruses existing in the spherical morphology of approximately 100 nm in diameter are grown in the cell culture system. However, influenza viruses isolated from the clinical specimens are believed to be predominantly filamentous particles. In addition; the internalization of the filamentous influenza virus particles is delayed according to their spherical particles [4].

Isolation of avian influenza virus (AIV) with the use of embryonated chicken egg (ECE) tends to be costly and requires much forethought concerning scheduling because embryos must be incubated 9–11 days prior to use [5]. In addition; the persistent propagation of AIV in ECEs has been shown to lead to the emergence of mutations in the HA glycoprotein [6] and the harvested ECEs may contain various microbiological contamination and residual endotoxin [7]. Finally, the ability of diagnostic laboratories to maintain a large volume of high-quality avian embryos can

be a limiting factor in isolation and propagation of influenza viruses. Other methods of AIV isolation and propagation have been explored, such as the use of cell cultures [8]. Influenza viruses can infect a variety of primary and continuous cell lines; however, most cells do not support productive viral replication. Currently, Madin-Darby canine kidney (MDCK) epithelial cells are widely used for viral studies since they support the growth and isolation of virus [9].

Due to increased demand for influenza viral vaccination, variable vaccine supply, and the threat of mutant influenza, cell culture-derived influenza vaccines from locally field isolates vaccines are being considered in addition to, or in replacement of, egg-based production so the present study was planned to propagate the low pathogenic strain H5N2 in different cell culture as looking further into the future, to vaccine preparation.

2. MATERIALS AND METHODS

2.1. *Virus:*

Egg adapted locally field isolate of low pathogenic avian influenza virus strain (H5N2); was kindly supplied by Veterinary Serum and Vaccine Research Institute (VSVRI) Abassia, Cairo.

2.2.*Tosyl-phenylalanyl-chloromethyl ketone (TPCK):*

It was supplied by Sigma Company and prepared according to the manufacturer directions then sterilized by filtration through 0.22μM mili-pore filter. TPCK reduced the chemotrypsin which is usually present in trypsin. It was added as 0.1 μg/ml for the maintenance medium used in propagation of the virus in MDCK cells.

2.3. *Cell cultures and Virus propagation:*

Primary chicken embryo fibroblast (CEF) cells were prepared from 9-11days of embryonated chicken egg [10].

The cell line of African green monkey kidney (vero), baby hamster kidney (BHK-21) and Madine Darby canine kidney (MDCK) cells were obtained from the Department of Pet. Animals vaccine resreach (VSVRI) Abassia , Cairo.

Ten successive passages were done for H5N2 virus in each type of cell; where in each viral passage 0.1 ml of purified virus was inoculated in the confluent monolayer cell culture previously prepared in cell culture tube. The demonstration, of cytopathic effect (CPE) was recorded and each viral passage was titrated using the tube method according to Singh et al [11] and the virus titer was calculated according to Reed and meunch formula [12]. The highest titer passages of H5N2 in both of MDCK and BHK-21were subjected to study the growth patterns , where infected cell culture tube subjected to titration of the cell free virus "CFV", cell associated virus "CAV" and total virus yield "TVY" in different hour interval post inoculation.

3. RESULTS

3.1. Propagation of LPAIV H5N2 in different cell cultures:

The onset of cytopathic effect (CPE) table (1) was detected 5 days post-inoculation (DPI) at the 1st passage in CEF while in MDCK ,VERO and BHK-21 were detected 4 DPI, 4 DPI and 3 DPI respectively ,then decreased to 2 DPI,1 DPI ,1 DPI and 1DPI at 10th passage. The complete CPE was detected at 7 DPI, 6DPI, 10 DPI, 6 DPI at 1st passage, while become 2DPI, 2DPI, 3DPI and 2DPI at 10th passage on CEF, MDCK, VERO and BHK-21 respectively.

3.2. *Titration of different passages of LPAIVH5N2 in different cells*:

The highest titer (table 2) in CEF was 106.5 TCID50 /ml at 10th passage while the titer in MDCK trypsin /TPCK treated was107.5TCID50/ml at 10th passage. The viral titer in VERO cells was gradually increased till reach to105.5 TCID50/ml at 10th passage, while the titer on BHK-21 was 107 TCID50/ml at 10th passage.

3.3. *Growth kinetic studies of LPAIVH5N2 in cell cultures*:

In MDCK cells, the cell free virus reached to the peak 72 hours post - inoculation (table 3) while the cell associated virus was 104 TCID50/ml 12 hour post inoculation then decline till reach to 10 TCID50/ml 72 hour post inoculation, but the total virus yield was 106.5 TCID50/ml and 107.5 TCID50/ml at 36 and 72 hours post inoculation. In BHK-21 cells the peak of virus titer was105, 103.5, 107 TCID50/ ml at 48 hours post- inoculation for CFV, CAV and TVY respectively.

Table 1. Propagation of different passage of LPAI H5N2 in different cells

Passage No.	CEF		MDCK(T/TPCK)		VERO		BHK	
	Onset of CPE	Complete CPE	Onset of CPE	Complete CPE	Onset of CPE	Complete CPE	Onset of CPE	Complete CPE
1	5*	7	4	6	4	10	3	6
2	4	6	4	6	4	9	3	4
3	4	7	3	5	3	8	2	3
4	3	6	2	5	3	8	2	3
5	4	5	3	4	3	7	1	2
6	3	5	2	4	2	6	1	2
7	2	4	1	3	2	5	1	2
8	2	3	1	2	1	3	1	2
9	2	2	1	2	1	3	1	2
10	2	2	1	2	1	3	1	2

*= Days post inoculation T/TPCK= trypsin /TPCK treated cells.

Table 2. Titration of different passage of LPAI H5N2 in different cell culture

Passage no.	Virus titer (log $_{10}$ $TCID_{50}$ / 1ml)			
	CEF	MDCK	VERO	BHK
1	1	1.5	2	>1
2	1.5	2	3	2
3	2	2.5	4	3
4	3	3	4	4
5	4	4	4	5
6	3.5	5	4.5	5.5
7	4	5.5	5	6
8	5	6	5	6.5
9	6	7	5.5	7
10	6.5	7.5	5.5	7

Table 3. Growth kinetic studies of LPAI H5N2 in different cell cultures

HPI	BHK-21			MDCK		
	A	B	C	A	B	C
1	0	Undil	Undil	0	≤ 1	≤ 0.5
2	0	Undil	Undil	0	1	1.5
4	0	1*	0	≤ 0.5	1.2	2.0
6	0	Undil	0	0.5	1.5	2.1
8	Undil	1	Undil	0.5	3.0	3.5
12	1	1	Undil	1.0	4.0	3.7
24	2	2	3	1.5	3.0	4.0
36	3	3	5	5.0	2.0	6.5
48	5	3.5	7	5.7	1.5	6.5
72	ND	ND	ND	6.5	1.0	7.6

*= virus titer (log 10 TCID50/ML), HPI:hours post inoculation, A=cell free virus, B=Cell associated C=Total virus yield, ND= Not Done, Undil= undiluted

4. DISCUSSION

The presented work was planned to culture low pathogenic strain H5N2 as a preliminary trails for vaccine preparation latter on , especially it is often possible to design program using available vaccine strain specially, if there was an outbreak with highly pathogenic H5N2 or incase of H5N1 prevalence, where H5N2 may protect against H5N1[13].

The inoculation and propagation of H5N2 in CEF, MDCK, VERO and BHK-21 (Table 1) were succeeded from 1st passage till 10th passage ,where the cells became rounded , some cells became elongated then lysis of cells occur. This CPE is not specific for influenza virus where other viruses make cell rounding and cell lysis. Anyhow, the ability of these cells to support viral replication with prompt onset of cell death suggests that they may provide adequate quantities of virus for early H5N2 isolation, titration and vaccine development. However, further studies examining the usefulness of other cells have to be undertaken.

Dealing with the result of titration of the virus as shown in table (2); CEF support growth of the LPAIH5N2, where Avian influenza A virus receptor were present on CEF cells [14], the presence of endogenous trypsin or trypsin-like enzyme in CEF cells support replication of LPH5N2 via enhancement of cleavage of HA0 [15]; that plays the most important pathway of viral replication cycle via viral attachment with cell membrane [16]. This indicated via the obtained result where; the titer was 10TCID50/ml in 1st passage then increased gradually till reach to $10^{6.5}TCID_{50}$/ml at the 10th passage.

MDCK trypsin /TPCK treated cells revealed acceptable result where the virus titer increase till reach to $10^{7.5}$ $TCID_{50}$/ml at 10th passage, that may be attributed to the fact that; MDCK cell line has expressed both receptors 2,3α sialic acid receptor and 2,6 α sialic acid receptor necessary for influenza viruses replication [17] while the addition of trypsin / TPCK to the media enhance the multiplication of the influenza viruses where TPCK inactivate the chemo-trypsin [18] that present with bovine trypsin in tissue culture media required for supporting and maintenance the cultured cell in-vitro. In addition; TPCK has another role, where it responsible for great enhancement of inoculated Influenza virus growth in MDCK cells by changing an amino acid residue in the stem region of HA2 subunit of the viral HA molecule; the mutation site located close to fusion peptide in HA trimer; that accomplish virus membrane fusion [19] during viral fusion to the infected cells. These results coincided with those obtained by van Wielink et al. [20] who found that MDCK cells supported the replication of most strains of LPAIV to high titres of about 108 TCID50 /ml and concluded that MDCK cells appear optimal for influenza virus production, since they propagate a wide variety of LPAIV strains to high titres without the need for virus adaptation.

Our results of LPH5N2 virus propagation in VERO was acceptable, where; Vero cell line has expressed both receptors 2,3α sialic acid receptor and 2,6 sailic acid receptor support replication [17]. The surprise result was yielded viral titer on Vero cell line was lower than MDCK where, Vero cells rapidly destroy exogenous trypsin by a factor secreted by Vero cells into the media [21], which limits the replication of influenza viruses with a non-cleavable HA to a single cycle. These results agreed with van Wielink ,et al [20] who recorded that Vero cells support the replication of most strains of LPAIV to lower titres compared to MDCK cells; Such a difference in virus yield between Vero and MDCK cells has been reported earlier[22, 23, 24].

The result of BHK-21 go hand in hand with those recorded by van Wielink et al, [20] who found that BHK21cells support the replication of most strains of LPAIV to high titres of about $10^{8}TCID_{50}$ equivalent/ml. It was a logical result that also agreed with

Govorkova et al [25] who observed low expression of sailic acid SA 2,6receptors on serum dependent BHK-21 cells and related it to the restricted replication of human influenza virus on these cells and the selection of receptor binding variants of the viruses that bind better to the SA 2, 3 receptors. This result disagree with those recorded by Moresco et al [26] who found that the most inefficient cultures for LPAIV growth were the BHK-21.

An important step in our work was study the growth curve of LP AIVH5N2in MDCK and BHK-21 that could be served in vaccine preparation, where the sequence of production of cell free, cell associated and total virus yield particle has been investigated. When looking to the data documented in table (3), we find that the eclipse phase was 8 hours post inoculation in BHK-21 but, in MDCK was 4 hours post inoculation. It was found that the best time for harvestation was 72 hours post infection yielding titer $10^{6.5}$ $TCID_{50}$/ml in cell free portion, 10^4 $TCID_{50}$/ml in cell associated portion after 12 hours post infection and $10^{7.5}$ $TCID_{50}$/ml at 72 post inoculation for MDCK, While for BHK-21the time for virus harvest was 48 hours post infection yielding titer 105 TCID50/ml in cell free portion and $10^{3.5}TCID_{50}$/ml in cell associated portion after 48 hours post infection. The total virus yield was harvested after 48 hours post inoculation with 10^7 $TCID_{50}$/ml. These findings support the replication of influenza AI- H5N2 virus to titers comparable to those in MDCK cells. These results suggest that Vero and BHK cells might serve as an alternative system for the isolation and cultivation of influenza A and may be useful for further studies in agreement with Sherrine [27] and Govorkova et al [22] who used Vero cells for isolation of AIV-H5N1. On the other side, Ozaki et al [28] and Lee et al [29] approved Vero cells as growth substrates for influenza.

It could be concluded that, the H5N2 virus was grown well in MDCK and BHK-21 cell as well as it was cell free virus with a high titer comparing to cell associated one.

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دراسات على خصائص نمو فيروس انفلونزا الطيور المنخفض الضراوة على خلايا الزرع النسيجي (H5N2)

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الملخص العربي

امكن امرار عترة منخفضة الضراوة (H5N2) من فيروس انفلونزا الطيور على انواع مختلفة من خلايا الزرع النسيجي (عشرة تمريرات) مثل خلايا اجنة الدجاج (CEF) وخلايا مادين دربي للكلى الكلبى المحور (MDCK) وخلايا كلى القرد الاخضر الأفريقي (VERO) وخلايا كلى اليربوع الذهبي (BHK–21).حيث تسبب الفيروس فى احداث التأثير المرضى للخلية (CPE) من اول تمريرة على كل الخلايا حيث شوهدت استدراه الخلايا واستطالة بعض منها وانتهاؤها بالسقوط وكانت أفضل الخلايا لنمو الفيروس هي خلايا مادين دربي للكلى الكلبى المحور والتي اعطت عيارية للفيروس107.5 عند التمريرة العاشرة بينما اعلى عيارية للفيروس على خلايا كلى اليربوع الذهبي وخلايا اجنة الدجاج على التوالي 105و107 عند التمريرة العاشرة ايضا وكذلك اظهرت النتائج ان الفيروس الحر خارج الخلايا يكون اكثر عياريه من الفيروس الموجود داخل الخلايا فى كل من خلايا مادين دربي للكلى الكلبى المحور وخلايا كلى اليربوع الذهبي.

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