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Research Article

The Effect of Multiple Passages of Egyptian HPAIV H5N1 on MDCK Cell Line on the Virus Titer and Mutation of HA Gene

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Abstract

H5N1 HPAI viruses were introduced in poultry sector in Egypt since 2006 and still circulated till now. Egyptian viruses are continually under evolution in the field and application of vaccination strategy as the first choice for AIV control so inactivated vaccine produced in egg system not suitable for the continuous evolution and incidence of unspurge mortalities and losses whereas egg adapted viruses aren't grow with the maximum level in cell culture. Therefore, the new technology for vaccine production in mammalian cell was attractive. MDCK has been used for AIV vaccine production for several years. Egyptian viruses are highly adapted for growth in ECE and the growth rate of these viruses not evaluated in cell line so we select 13 isolates of Egyptian HPAIV represent 2 clade present on the field based on phylogenetic analysis are passaged in MDCK cell line for ten serial passages. The growth capacities of these viruses after passages on cell line were measured by Haemagglutination test after each passage, quantitative RRTPCR test and TCID50 after 10th passage. The virus titer after passages is between 7 to 8.5 log 10 TID50. Genetic and antigenic properties of the passage viruses are stable from 1st passage till 10th passage. Sequence analysis of HA gene of the original viruses identified different point mutation in HA gene which characteristic to clade 2.2.1 viruses and may be lead to increase biological fitness and virus load of this viruses in mammalian cells. In conclusion, MDCK cell culture are suitable for production of tissue culture adapted viruses with genetic and antigenic stability after multiple serial passages in the MDCK cell which is a promising way after that for AIV tissue culture based vaccines.

Keywords: *Highly Pathogenic Avian Influenza Virus; MDCK; Antigenic Characterization; Sequencing and rRT-PCR*

Introduction

AIV have wide spread geographical distribution around the world, Egypt become endemic with HPAI virus after circulation of the virus in the poultry sector at mid-February 2006 [1]. Orthomyxoviride was the family name of avian influenza viruses and type A viruses were circulated in poultry. Surface glycoproteins either heamaglutinine or neuraminidase are characteristic for viruses and by this protein we can classification of viruses to several types, up till now there are 16 HA subtype and 11 NA subtype circulated in bird and 2 subtypes H17N10 and H18N11 isolated from bats [24]. HA protein is the most abundant protein in the surface of the virus and the most effective one due to its contain RBD responsible for virus attachment and antigenic sites related with the immunity and any antigenic drift in this site may be lead to escape mutant viruses from immunity so antigenic stability of viruses is important for immunity, infectivity and host transmission of viruses [12]. Influenza virus infection basically depend on the cleavability of HA protein [32]. Pathogenicity of AIV related with many factors depend on the virus and host at the same time so the notified viruses as HP viruses was H5 and H7 viruses according to European union [27]. Highly pathogenic avian influenza viruses subtype H5 and H7 have multiple basic amino acids in their cleavage site which can be cleaved by furine which present in different tissue while LP viruses HA was cleaved only by trypsin restricted to respiratory system [20]. For infectivity and cell fusion of viruses HA protein must be cleaved to 2 parts HA1 and HA2 [32]. The Madin Darby Canine Kidney (MDCK) cell line was originally isolated in 1958 [10]. From last decade in past century MDCK cell line was used in propagation

and isolation of avian influenza viruses, after modification of the system by adding trypsin to culture fluid viruses produce clear CPE and efficient growth with high yield [22]. Other cell line such as PMK and VERO cell plus MDCK cell are now considered the promise new technology and gold stander technique for propagation of influenza virus's seed vaccine [4]. AIV type A viruses grow in MDCK cells with high titer and virus load [17]. CPE produced on MDCK cell line after infection by both path types of AIV either HP or LP viruses in the absence of trypsin [16]. Cell culture growing HPAI virus retains the antigenic properties of the parent strain in original sample [11]. Post isolation identification based on the demonstration of the RNA of the virus in harvested cell culture material can be performed by modern fast diagnostic technique such as PCR [19]. Vaccination strategy is the most applied in control of AI virus infection, Egg adapted vaccines alone cannot cover the demand of vaccination, so cell culture based vaccines are necessary and the urgent need to address the continuous development of viruses, 2 cell line Vero and MDCK cells have been licensed for manufacturing influenza vaccines by WHO but only MDCK cell line which used in poultry vaccination system while VERO cell licensed only for human vaccine. Continues passage of AIV in cell line lead to increase of the virus load and titer with genetic and antigenic stability of the viruses which not adverse with vaccination production system [25]. MDCK cell line was preferred cell line for establishment of tissue culture system and replaced egg based system either in diagnoses and vaccine production is come back to antigenic and genetic stability of viruses after propagation and passaging in cell [21]. This study was conducted to adapt the Egyptian viruses to grow efficiently in the MDCK cell line with antigenic and genetic stability which the main potency marker of influenza vaccines.

Material and Methods

Viruses: Thirteen viruses were propagated and isolated in virology unit in RLQP, Egypt as shown in table 1. They were isolated from swab samples collected from birds (5 from chickens and 8 ducks) in the farm sector from 2008 to 2009 (Table 1). The original samples were collected in the lab during active surveillance for monitoring of avian influenza virus in Egypt depend on ministerial decree approved to the lab.

Cells: MDCK cells were provided from Vacsera Company, Egypt. The clone of cells was 20 passage clone cell and passage in the lab for 2 passages and after that used in the trial. The cell was cultivated in 6 well tissue culture plate with adding of GM (Eagle MEM) containing 10% fetal bovine serum and antibiotic according to stander and incubated at 37°C with 5% CO_2 until produced confluent sheet.

Cell infection by viruses: The confluent sheet cells in tissue culture plate were washed twice with phosphate buffered saline (PBS) and infected with 1000 virus particles from each virus. The plate was incubated for 2hrs in 37°C with 5% CO_2 for virus attachment with the cell. After that we discard the supernatant fluid and adding 1000 ul of MM containing 2% of fetal calf serum to each well in the plate and incubate it at 37°C with 5% CO_2 for 3 days. Uninfected confluent sheet well remain as negative control in each plate.

Serial Passages of viruses in cell line: The viruses were passaged for 10 serial passages in MDCK cell line through preparation of tissue culture plate with confluent sheet and infected by viruses and incubated at 37°C with 5% CO_2 for 72hr and examined every 12hr for CPE and collection of tissue culture fluid 72hr pi and tested by HA test and positive viruses inoculated again for 10 times [2,29].

Haemagglutination assay: The presence of H5 virus in collected tissue culture fluid was examined by HA plate test after each passage by using chicken RBCs 1% and PBS and HA test was applied according to stander protocol [13].

Quantitative Real time RT-PCR: Quantitative RT-PCR used for confirmation of H5 viruses in tissue culture fluid after 5th and 10th passage for confirmation the growth of H5 viruses. In brief RNA extraction was performed according to the manufacturer's recommendations using the QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany). Genome amplification, detection and analysis were performed in a Stratagen MX3005P machine (Agilent, California, USA). The extracted RNA also tested against any contamination with other HA agent like NDV, H7 and H9 viruses.

Virus Infectivity Titration, TCID50 and Antigenic characterization of passage viruses: TCID50 was calculated of each wild virus and tissue culture growing virus after 10th passage using MDCK cells. TCID50 was used for evaluation of growth kinetic difference between

the wild virus and tissue culture propagated one by comparison between the infectivity of viruses before and after passaging at different time point 12hr, 24hr, 36hr, 48hr, 72hr and 96hr pi. The collected tissue culture fluid from each time point of each virus was diluted 10 fold serial dilution and each dilution inoculated in well of 96 tissue culture plate and incubated for 72hr at 37°C with 5% CO_2. CPE was examined and TCID50 was calculated by the method of Reed and Muench [8]. The antisera were prepared in the RLQP animal facilities. 13 Egyptian H5N1 viruses from different clades were inactivated by 0.01% formalin (Merck, Germany). Montanide® ISA 720 (Seppic Inc., France) as adjuvant was added to the antigens according the manufacturer recommendations. Three-week-old SPF chickens were injected intramuscular and were euthanized three weeks later. Antigenic test of 10th passage virus against homologous antisera of wild one by HI test and cross HI test between the viruses of 2 clades according to standard protocol [13].

Sequence and phylogenetic analyses

Using One-Step RT-PCR Kit and generic primers [6], HA gene was successfully amplified for all viruses. Amplicons were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequences were obtained using an ABI Big Dye Terminator v.3.1 Sequencing Kit (Perkin-Elmer, Foster city, CA) in Applied Biosystems 3130 genetic Analyzer (ABI, USA). The obtained sequences were submitted to a BLAST search to confirm the high similarity with other H5N1 viruses in GenBank [1]. Sequences generated in this study are available in the GenBank and their accession numbers are provided in table 1. Phylogenetic relatedness of sequences generated in this study to other A/H5N1viruses from Egypt were done by retrieving relevant gene sequences from GenBank. Nucleotides and deduced amino acids (aa) were analysed using MAFFT [6] and further edited by Bio Edit 7.1.7 [5]. Maximum likelihood trees were constructed after selection of the best fit model and Mr Bayes as implemented in Topali v.2 software [13]. The phylogenetic tree was further edited by Inkscape 2.0 (Free Software Foundation, Inc., Boston, USA).

Result

Sequence and phylogenetic analyses: Phylogenetic analysis of HA gene of original viruses indicated the viruses present in the study were present in 2 clades 9 of them present in clade 2.2.1 and another 4 viruses present in clade 2.21.1 (Figure 1). Sequence results pointed out the HA gene of 2 clades of the viruses have some point mutation which proven through aa identity between the viruses which ranged from 99 to 91.7 and clade 2.2.1 viruses have characteristic mutation reflect on virus fitness and propagation with high efficient in mammalian cell.

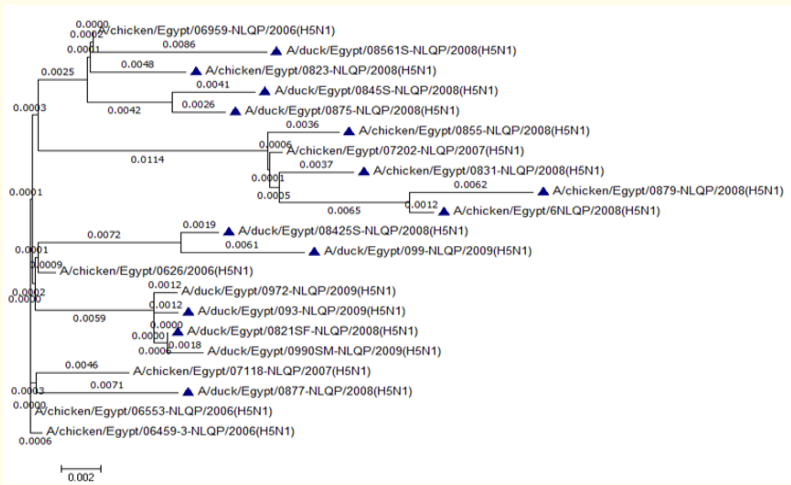

Figure 1: *Phylogenetic relatedness of the hemagglutinin (HA) gene of A/H5N1 used in this study Maximum likelihood mid-point tree and Phylogenetic relatedness of sequences generated in this study to other A/H5N1viruses from Egypt was done by retrieving relevant gene sequences from GenBank. Multiple sequence alignment was done using MAFFT and BioEdit and was further edited manually. Maximum likelihood mid-point tree was constructed using MrBayes after selection of the best fit model as implemented in Topali v.2 software (Milne, et al., 2009). The phylogenetic tree was further edited by Inkscape 2.0 (Free Software Foundation, Inc., Boston, USA).*

Virus propagation on cell line: 13 viruses present in the study have the ability for infection of MDCK cells and production of CPE within 72hr pi in the absence of trypsin (Figure 2A, B and C). CPE were noticed in the cell from 24hr pi up to 72hr pi and the size of CPE were increased in parallel to passaging of viruses in cell. At the first few passages until 4th passages the large plaque formation can be noticed at 5to 6 days pi while in last passages this CPE can noticed between 3 to 4 days post infection of the cells, the uninfected cells (negative control) appear normal during period of incubation (Figure 2D). CPE percent also increased in parallel to no of passages as we can notice the CPE percent about 60 to70 in first 5 passages with an increase ratio about 10% in clade 2.2.1 viruses more than clade 2.2.1.1 viruses whereas the percent of CPE reach to 80 to 95% with an increase ratio about 15% between viruses of 2 clades and CPE detected 24hr pi and 36hr pi at 5th passage in clade 2.2.1 and 2.2.1.1 viruses respectively and at 10th passage detected at 12hr pi and 24hr pi in clade 2.2.1 and clade 2.2.1.1 viruses respectively with significance difference ($P < 0.05$) (Figure 3).

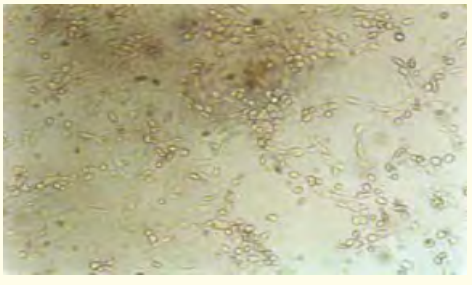

Figure 2a: *Detachment of the cells 24hr post inoculation at last 4 passages of the viruses.*

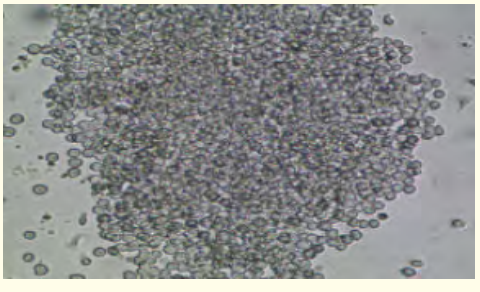

Figure 2b: *Rounding of cells 48hr post inoculation at last 4 passages.*

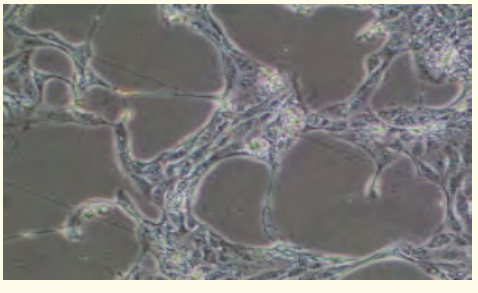

Figure 2c: *Large plaques of the cells 72hr post inoculation with local HPAI virus at last 4 passages.*

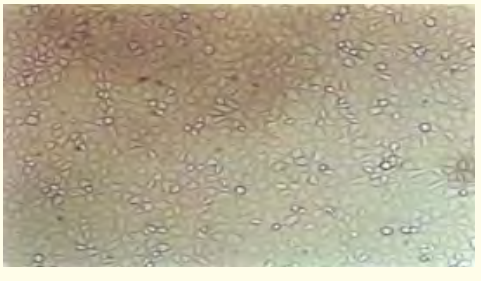

Figure 2d: *Uninfected control MDCK cells 72 hr Post inoculation (negative control).*

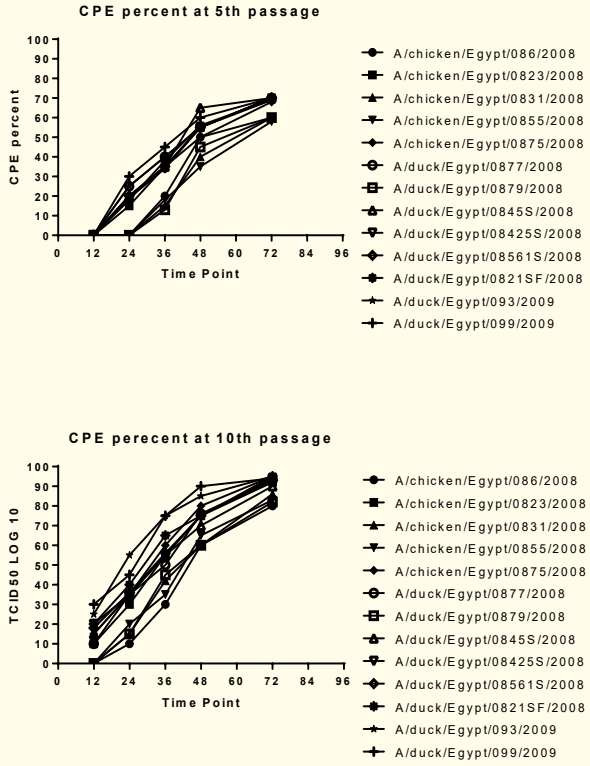

Figure 3: *Explained the difference of CPE percent between tissue culture growing viruses at 5th and 10th passage on MDCK cell line.*

Detection of virus growth in cell line after each passage: Tissue culture fluid was collected 72hr pi in each passage and tested by HA test for detection of the virus and the result revealed that 123 viruses have the ability to grow in MDCK cell line with continence rise in HA titer after each passage which start from 2 to 3 log 2 at 1st passage and reach to 8 log 2 in 10th passage with slight increase in clade 2.2.1 viruses about 2log more than clade 2.2.1.1 viruses (Figure 4).

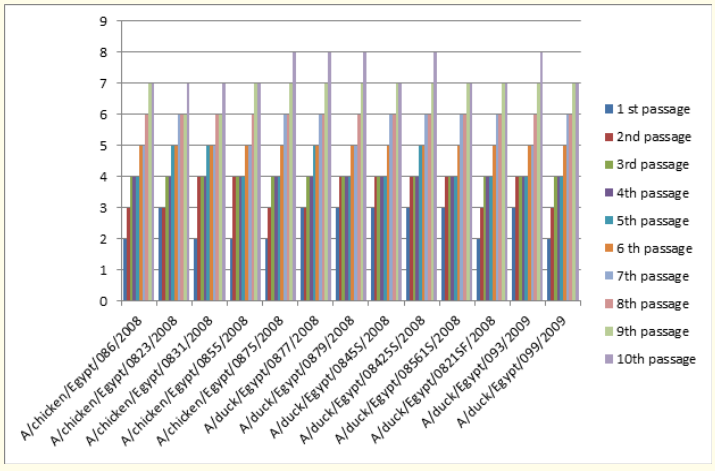

Figure 4: *Titration of AIV after each passage on MDCK cell line by HA assay explain the titer of each virus at 1st to 10th passage log 2.*

Quantitative real time PCR result: PCR test was performed on tissue culture fluid of all of viruses after 5th and 10th passage for confirmation the growth of H5 only and the kinetic of growth of the viruses and results proven the growth of H5 viruses only without any contaminant in tissue culture fluid and the growth kinetic of viruses increased which confirmed by the ct values reflect the no of gene copies as we see the ct value at 5th passage ranged from 22 to 28 and after 10th passage ranged from 25 to 18.

Comparison the infectivity of tissue culture growing virus and wild one: Tissue culture fluid collected at different time point from 10th passage of each viruses and compared with the parent viruses by TCID50 and result revealed the growth of viruses with increase of virus load clearly present at 10th passage with 2 to 3 log difference between them and growth kinetics of clade 2.2.1 viruses was higher than clade 2.2.1.1 viruses with about 1.5 log and first detection of cell culture growing virus at 24hr pi in clade 2.2.1 and 36hr pi in clade 2.2.1.1 whereas wild viruses was detected in tissue culture fluid 36hr pi in clade 2.2.1 viruses and 48hr pi in clade 2.2.1.1 viruses with significance difference ($P < 0.05$) (Figure 5).

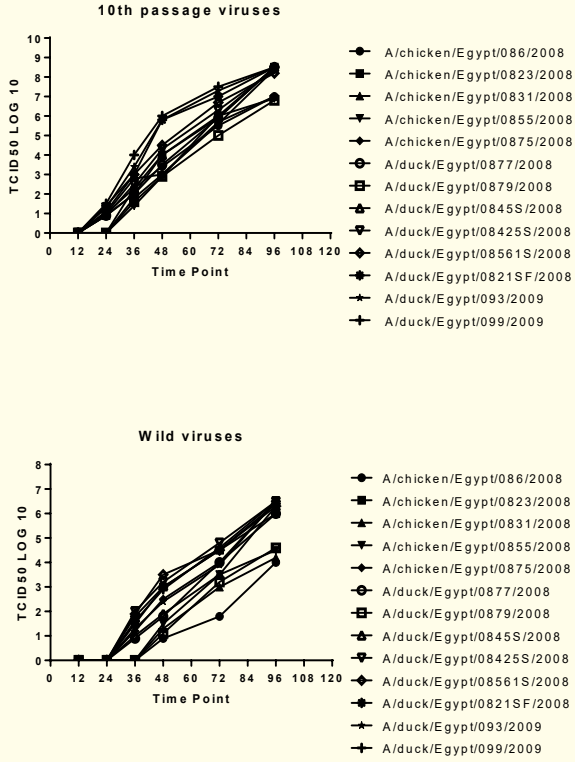

Figure 5: *Explained the difference in infectivity between the tissue culture growing virus after 10th passage and wild viruses by TCID50 calculated for the 13 viruses at different time point.*

Antigenic and genetic stability of HA gene after passages: Antigenic result revealed the antigenic variation between the 2 clades of viruses which about 3 to 4 logs difference between the viruses of 2 clades while the 10th passage growing virus is closely related to the wild one (Figure 6). Comparison between sequence analysis of HA gene of tissue culture growing viruses after 10th passage and wild viruses revealed absence of mutation between the viruses on the level of HA gene which support the use of MDCK cell line in production of tissue culture stable adapted seed viruses (Table 1).

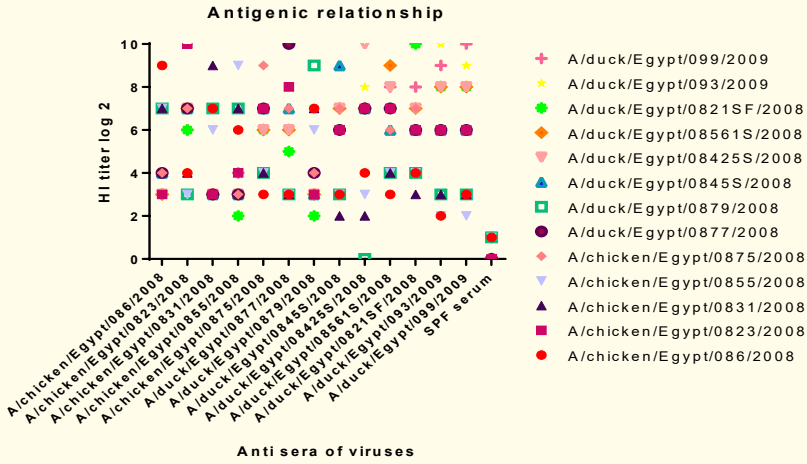

Figure 6: *Antigenic characterization of viruses present in the study after 10th passage using AS of wild viruses and antigenic relationship between s clades of viruses based on haemagglutination inhibition test log2.*

No.	Virus	Clade	Governorate	HA accession no	Sequence results*	
					5th passage	10th passage
1	A/chicken/Egypt/086NLQP/2008	2.2.1.1	Qena	EU717857	0/1705	0/1705
3	A/chicken/Egypt/0823NLQP/2008	2.2.1	Al-Gharbya	ACR56227	0/1705	0/1705
4	A/chicken/Egypt/0831NLQP/2008	2.2.1.1	Al-Qalubia	AR56228	0/1705	0/1705
5	A/chicken/Egypt/0855NLQP/2008	2.2.1.1	Al-Gharbya	ACR56235	0/1705	0/1705
6	A/chicken/Egypt/0875-NLQP/2008	2.2.1	Mattrouh	ACR56240	0/1705	0/1705
7	A/duck/Egypt/0877-NLQP/2008	2.2.1	Luxor	ACR56242	0/1705	0/1705
8	A/duck/Egypt/0879-NLQP/2008	2.2.1.1	6th of October	GQ184238	0/1705	0/1705
10	A/duck/Egypt/0845S-NLQP/2008	2.2.1	Al-Menofya	ACR56249	0/1705	0/1705
11	A/duck/Egypt/08425SNLQP/2008	2.2.1	Al-Menia	ACR56255	0/1705	0/1705
12	A/duck/Egypt/08561SNLQP/2008	2.2.1	Al-Sharqia	ADD 21352	0/1705	0/1705
13	A/duck/Egypt/0821SFNLQP/2008	2.2.1	Al-Menia	ACR 56248	0/1705	0/1705
14	A/duck/Egypt/093-NLQP/2009	2.2.1	6th of October	ACX 31992	0/1705	0/1705
15	A/duck/Egypt/099-NLQP/2009	2.2.1	Al-Gharbya	GU002673	0/1705	0/1705

Table 1: *Data of selected 13 viruses in the trial and sequence analysis of HA gene of HPAIV of all viruses revealed absence of any changes or motives after 5th or 10th passages on MDCK cell line in comparison with the original HA viruses sequence*

**Substitution/ Total*

Discussion

Egg adapted viruses for production of seed virus and diagnostic material synthesis faces some troubles due to the genetic instability of viruses after serial passages on ECE to obtain the highest virus load from this viruses to be ready to use in manufacturing of diagnostics and vaccine production, so implantation of another system for adaptation of viruses by cell line and production of cell adapted viruses is the first demand in poultry field today. To avoid the inconvenience, MDCK cell cultured adapted virus is considered the best way for this

technology due to the only licensed cell line system for poultry vaccine production and replacing ECE system was MDCK cell line [15]. In comparison between both systems for vaccine production either ECE system or cell culture system has the same protection level in both human and avian [26]. The application of cell line such as MDCK or VERO cell in production of vaccine is advocated safer more than the uses of ECE and can be alternate it in vaccine production [23]. Vero cell adapted seed virus induces mutations that impair virus stability, leading to decreased immunogenicity of this seed virus which has adverse effect on vaccine [15]. For overcome the limitation of egg system modern technology was creative for vaccine production such as mammalian cell line or insect cell production system [3]. Different types of cell line support the growth of virus and especially MDCK cell has the opportunity for virus propagation in absence of any extraneous additive making it the perfect and safety method for vaccine production [11]. With all in the above MDCK cell express both types of receptors on its surface avian and human receptors [16]. In this study we adapted the Egyptian HPAIV to grow on MDCK cell line for production of cell based seed virus suitable for vaccine production after 10 passages on cell with obtaining the high yield and virus load without addition of trypsin which confirm the preservation of genetic and pathogenic characteristic of these viruses after passaging. Virus infection to the cell related with the cleavage of HA protein of the virus so HA protein is the most important protein on the surface of the virus and responsible for attachment with the cell due to the presence of RBD on globular tip of HA [18]. Our obtained data describe the difference between the tissue culture adapted virus and wild one through measurement the growth yield and infectivity of the viruses and we find that tissue culture adapted viruses after 10 serial passages on MDCK cell line were have difference in TCID50 about 3 logs higher than wild viruses and CPE percent increased each passage until reach 95% at 10^{th} passage. The growth yield of each virus was measured after each passage by HA test and results revealed the gradual increase in virus yield after each passage and reach to 8log 2 at 10^{th} passage in clade 2.2.1 viruses which matches with the ct values of the viruses whereas ct values describe the gene copy of the virus and the 10th passage viruses have high gene copy more than wild viruses which can be used as indicator for virus growth with high yield in cell line. Avian Influenza viruses were highly mutated viruses and percent of incidence of antigenic drift was high and may be present in globular head of HA gene and is inconsistent with antibodies [29]. The main effect of antigenic drift is giving the virus an escape mechanism from immune system of the bird and can attached to the receptors and entrance the host cell making infection [31]. Therefore, avian influenza viruses may gain new mutation which give it the ability to penetrate the immune system and capability for replication in mammalian cell with high growth rate and possibility of transmission to human [28]. The result indicate the difference between viruses in growth yield even after passaging in cell line and viruses of clade 2.2.1 have high growth rate more than viruses of clade 2.2.1.1 and this difference is clear in results of testing parameter for growth yield used in this study. Refer to sequence analysis of wild viruses data of sequence tell us about the existence of 3 point mutation on HA gene of clade 2.2.1 (N94D, N117D and D154N) which studied before in Egyptian strain and presence of this mutation increase the affinity of viruses for replication in mammalian cell [28]. MDCK can support AIV replication to sufficient yield without any effect on nucleotide sequence of HA gene [7,30]. Incidence of amino acid substitution on HA protein during serial passage not found so the improvement in growth rate of viruses and increasing in the virus load in the cell without any alteration on genetic and antigenic properties of the viruses support application of this technology in preparation of seed viruses. This result suggest the improvement in growth of viruses after serial passage on MDCK cell line related with percent of receptors on the surface of the cell and point mutation present in viruses. Indeed, the study demonstrates that the suitable use of MDCK cells for preparation of adapted cell culture seed viruses with antigenic stability which support the using of these type of cells in the septic propagation of HPAIV for antigen and vaccine production and the finding showed that MDCK cell support the growth of HPAIV without trypsin with minimum quantity of serum Making it the most appropriate type for influenza diagnostics and vaccine production.

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