

Effect of Mutations in Highly Pathogenic Avian Influenza H5N1 Viruses on the Isolation in Different Cell Culture in Comparison with Newly Circulated H5N8 Viruses in Egypt

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Abstract

HPAIV H5N1 was circulated in Egypt since 2006 with recent introduction of H5N8 virus at the end of 2016. AIV has special virus Tropism and infectivity in different isolation host. There for we study the growth kinetics for 9 H5N1 viruses and one H5N8 virus in 3 different tissue culture systems (MDCK, CEF and VERO) and detection the potential bounding to mammalian host. Growth kinetics of cell culture growing viruses was estimated by different parameter as TCID₅₀, CPE percent and HA activity of viruses. Growth kinetics level of Clade 2.2.1.2 and new isolated H5N8 is the highest with early detection of the virus in cell within 1st 24 hr in comparison to another 2 clades of H5N1 viruses. MDCK support high growth kinetics for all clades of virus between 3 types of cell. Antigenic characterization of the viruses supported the results of the phylogenetic analysis. Multiple peculiar mutations were characterized in the Egyptian H5N1 and H5N8 viruses. The Egyptian AI viruses preferentially bound to avian-like receptors rather than human-like receptors. MDCK cell line was suitable isolation host to replace egg isolation system with antigenic and genetic stability. This study describing the growth kinetics of HPAIV H5N1 and H5N8 in various cell lines is an informative study to understand influenza virus establishment and evolution in regard to preventive measures and cure.

Keywords: HPAIV; Cell culture system; Virus evolution; Growth kinetics; Antigenic relationship and genetic stability

Introduction

The continuous evolution of HP avian influenza virus H5N1 in poultry sector in Egypt with introduction of H5N8 virus from migratory bird and also recorded in domesticated ducks is an important issue in the field so study the mechanism of tropism and growth level in different cell culture system may be directed us to predict the virulence manner of these viruses and renew our isolation system for production of highly stable tissue culture adapted virus and detect the possibility to bound to mammalian receptor. Since 1996/1997, highly pathogenic avian influenza virus (HPAIV) of H5N1 subtype (A/H5N1) diversified into 10 phylogenetic clades (designated clade 0 to 9) based on the sequence homology of the hemagglutinin (HA) gene. Viruses in clade 2.2 were widespread in poultry in many countries in Asia, Europe and Africa [1,2]. HPAI virus H5N1 is endemic in Egypt and lead to serious outbreaks in poultry sector from 1st record of virus in 2006 and has caused occasional infections in human. Phylogenic analysis of 1st record Egyptian H5N1 viruses are belonged to clade 2.2.1 and with continuous evolution of the viruses in the field lead to appearance of different clade 2.2.1.1 with antigenic drift variant which has been sprouted and spread in vaccinated chicken at the first of 2008 and decline at 2011 [3]. As a result of continues circulation of virus in the field new distinct cluster 2.2.1.2 become appeared in 2012 and gained predominance since summer 2014 and caused high mortality rate in poultry holdings in late 2014 [3]. The first evolution of Egyptian viruses could be detected in a farm sector with low incidence in the house hold sector, while the last evolution of Egyptian viruses and appearance of the clade 2.2.1.2 viruses gained predominance and circulated in all poultry sector and have ability to infect human [4]. At the end of 2016 newly circulated HPAIV subtype H5N8 isolated from migratory birds in Egypt, documenting its introduction to Africa. Avian influenza virus is belonging to *orthomyxoviridae* type A viruses, up till now 16 heamagglutinine and 9 neuramidase has been detected in poultry and 2 new types H17N10 and H18N1 have been detected in bats [5]. Most of theory of virus infection was built on surface projection

glycoprotein Hemagglutinin and Neuraminidase. As mentioned before HA protein responsible for attachment and entrance of virus inside the cell and NA protein help in release of the virus from the cell and spread of infection [6]. Madin-Darby Canine Kidney (MDCK) and African Green Monkey Kidney (Vero) cells are much advocated presently as a safer alternative to chicken embryonated eggs in virus isolation and propagation. Information on the growth of influenza virus in cell lines provides insights in understanding the patterns of virus replication [7]. MDCK grown influenza virus was antigenically equivalent to egg grown influenza viruses. A thorough comparison of virus growth in cell lines might help to develop an optimized vaccine production strategy, as well as to assess quality differences concerning the virus strains and antigen produced [8]. Stability of propagated viruses is very important for implantation of cell culture system in propagation of avian influenza viruses and vaccine production so propagation in CE and MDCK cells have a similar effect on virus stability but virus propagation in Vero cells induces mutations that impair virus stability. MDCK cell line remains the most widely used cell line in influenza virus research due to its high susceptibility to various influenza viruses [9]. Currently, there are different clades of influenza virus H5N1 circulated in poultry sector and the most applicable control measure was the vaccination so in addition to influenza vaccines, Vero cells have been licensed for other human vaccines but MDCK cells are only licensed for influenza vaccines [5].

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HA protein contain RBD and antigenic sites so the influenza virions undergo mutation and antigenic drift to escape from the immune system and induce the infection so maybe point mutation in HA gene can led to decrease virus propagation in egg or tissue culture system. Receptor binding sits in HA gene play the major role in host specificity, tissue tropism, pathogenicity and virus transmission between the hosts [10]. Polymerase complex subunits play a role in virus pathogenicity and efficient viral growth in mammalian cell [11]. In present study we exploited the genetic and virological approaches to clarify the possibility of deviation to mammalian receptors and detection the growth yield of these viruses in different cell line without addition of any extern use material and minimum amount of serum and stability of the virus on cell line which can replace the egg isolation system.

Materials and Methods

Virus

Eight HPAI H5N1 viruses isolated in RLQP represent 3 circulated clades of the Egyptian HPAIV H5N1 viruses from 2006 to 2015 and one H5N8 virus from migratory bird. The detailed data of the viruses present in Table 1. Six H5N1 viruses were isolated from poultry farm with vaccination history and 2 viruses from backyard sector and vaccination history not found for these 2 viruses A/chicken/Egypt/15S40/2015 and A/chicken/Egypt/15S75/2015 and H5N8 from migratory bird A/ Common coot/Egypt/CA285/2016.

Virus Infectivity on ECE

The viral infectivity of each strain was determined by serial titration in 10-11-day-old embryonated eggs, and was expressed as 50% of the egg infective dose (EID50)/ mL using the method reported by Reed and Muench [12].

Cells

1×10^6 MDCK cells, VERO cells obtained from (vacsera comp) and CEF cell were maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotic concentration according to stander procedure in 6 well micro plates. The confluent cells were washed twice with PBS and infected with 100 μ l of 4 log 10 EID50 from each virus and incubated 1 hr at 37°C with 5% CO₂ and after that discard the supernatant fluid and adding 1 ml MD containing 2% serum and incubated at 37°C with 5% CO₂ environment. Supernatant fluid collected at different time point 12 hr, 24 hr, 36 hr, 48 hr, 72 hr, 96 hr and 120 hr post infection with collection of cells in separated tubes at 120 hr pi and stored at -7°C until titration by HA test. One uninfected well in each plate was considered as negative control [13].

Growth kinetics by Real Time-PCR

Virus detection and quantification were conducted in a Taqman

qRT-PCR, targeting the influenza type A matrix (M) gene [14]. Briefly, 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). An absolute quantification of the AIV matrix gene specific RNA was achieved relative to a standard curve, based on the tenfold dilution of an in vitro transcribed RNA template of the inoculated viruses. The current detection limit of the qRT-PCR is 2.3 copies. A cut threshold (Ct) value of 40 was selected as the cut-off between positive and negative results; therefore samples with a Ct higher than 40 were considered negative for AIV. The results were expressed as the number of M gene copies per ml of collected tissue culture fluid. The tissue culture fluid collected within different time point 12 hr, 24 hr, 36 hr, 48 hr and 72 hr pi.

Haemagglutination assay

The haemagglutination assay was performed using 1% chicken red blood cells (in PBS, pH 7.2) in round-bottom 96-well plated by a standard technique [15]. 25 μ l PBS dispensed in HA plate then 25 μ l of supernatant tissue culture fluids from time point 12 hr, 24 hr, 36 hr, 48 hr, 72 h, 96 hr and 120 hr pi add to 1st row and serial dilution to the last row after that 25 μ l of RBCs 1% add to the plate and incubated for 30 min in room temperature.

TCID50

The infectivity of each virus calculated at different time point 24 hr, 36 hr, 48 hr, 72 hr and 96 hr by collecting the cell supernatant fluid and stored at -70 c until titration. Each collected time point virus diluted 10 fold serial dilutions from the 1st to the 10th dilution in MEM Eagle media and inoculated 100 μ l of each dilution in 8 well in 96 tissue culture plate containing confluent sheet and incubated 1 hr at 37°C with 5% CO₂ and after that discard the supernatant fluid and adding 500 μ l MD and incubate for 72 hr at 37°C with 5% CO₂, after that calculation of TCID50 by read and Muench through detection of CPE in inoculated wells. 2 row of confluent uninfected well passed in each plate as negative control.

Antigenic characterization

Antigenic characterization of A/H5N1 and H5N8 in this study was done using the haemagglutination inhibition (HI) test according to the OIE protocol [15]. The antisera were prepared in the RLQP animal facilities. 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.

No.	Virus	Clade	HA accession no	EID50 log 10 per ml
1	A/chicken/Egypt/06459NLQP/2006	2.2.1	EU372944	7.5
3	A/chicken/Egypt/07175NLQP/2007	2.2.1	GQ184215	8
4	A/chicken/Egypt/07202NLQP/2007	2.2.1.1	EU496389	7
5	A/chicken/Egypt/0831NLQP/2008	2.2.1.1	GQ184223	7.5
6	A/chicken/Egypt/1055NLQP/2010	2.2.1.1	HQ198268	7
7	A/ chicken /Egypt/116ADNLQP/2011	2.2.1.1	JN807843	6.5
8	A/ chicken /Egypt/1540SNLQP/2015	2.2.1.2	EPI573336	8
9	A/ chicken /Egypt/1575SNLQP/2015	2.2.1.2	EPI573317	8.5
10	A/ Common coot/ Egypt/CA285/2016	2.3.3.3	EPI 1018198	6.5

Table 1: Explain the detailed data of the viruses used in the study.

Sequence and phylogenetic analyses

Using One-Step RT-PCR Kit and generic primers [16], 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. 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/H5N1 viruses from Egypt was done by retrieving relevant gene sequences from GenBank. Nucleotides and deduced amino acids (aa) were analysed using MAFFT [16] and further edited by BioEdit 7.1.7 [10]. Maximum likelihood trees were constructed after selection of the best fit model and Mr Bayes as implemented in Topali v.2 software [15]. The phylogenetic tree was further edited by Ink scape 2.0 (Free Software Foundation, Inc., Boston, USA).

Results

Phylogenetic analyses

The eight tested viruses were present in 3 different clades of avian influenza viruses according to phylogenetic analysis of HA gene 2 viruses of them in clade 2.2.1.2, A/chicken/Egypt/1575S/2015 and A/chicken/Egypt/1540S/2015 and 2 viruses were present in clade 2.2.1 A/chicken/Egypt/06459/2006 and A/chicken/Egypt/06175/2006 and the remaining 4 viruses were present in clade 2.2.1.1 as data present in phylogenetic tree in Figure 1. Phylogenetic analysis of HA gene sequences revealed that EG-CA285 virus is clustered with clade 2.3.4.4b, along with the recent viruses widely distributed throughout Europe [17].

Sequence analyses

Sequence data of the tested viruses revealed that amino acid substitution on HA. Mutation present in HA gene observed in conservative region and near to RBS and glycosylation site with another present in stalk domain of HA2 which may be have some effect on the virus binding affinity to mammalian cells. Clade 2.2.1.2 viruses have the highest no of mutation followed by clade 2.2.1 and at the end clade 2.2.1.1. While H5N8 virus has 3amino acid assignment differences in the HA protein (Figure 2A and 2B).

Virus titration on ECE

The original tested viruses have EID₅₀ ranged from 6.5 to 8.5 log₁₀ after titration on SPF ECE with difference between the clades of viruses. The two viruses present in clade 2.2.1.2 have high titter with 1log more than other viruses as present in Table 1.

Determination of CPE in cell line

The replication of tested viruses was observed by the presence of CPE on different cell line. All of cell types were susceptible to HPAIV and produce CPE within 72 hr but MDCK cells appear to more sensitive for influenza virus infection as can detect CPE within 24 hr pi with maximum percent of CPE 72 hr pi while another 2 types of cell within 36 hr pi with maximum percent at 72 hr pi. Clade 2.2.1.2 viruses poses the highest percent of CPE in all cell lines and the percentage of CPE in different cell line were 90.99, 86, 9 and 80, 6 MDCK, CEF and VERO cell respectively 72 hr pi while H5N8 virus poses CPE percent about 89.8, 85.6 and 20.3 in MDCK, CEF and VERO cell respectively

with significance difference ($P>0.05$) (Figure 3A and 3B).

Replication of viruses in different cell line

HA titer calculated from the collected supernatant TC fluid harvested at different time point revealed the viruses of clade 2.2.1.2 early detected in cell as can be detected within 12 hr pi followed by clade 2.2.1 and 2.2.1.1 24 hr and 36 hr respectively. The highest titter appear in viruses of clade 2.2.1.2 which reach 7 log₂ at 120 hr pi in MDCK cell. Whereas CEF cell give low titer ranged from 2log₂ to 5log₂ and virus can be detected in clade 2.2.1.2 within 24 hr pi and another clades detected within 36 hr pi and VERO cell have the same time detection as CEF but with low titer which ranged from 2log₂ to 4log₂. Clade 2.3.4.4 virus poses high HA titer in MDCK cell line within 120 hr pi about 6log₂ and can be detected 24 hr pi followed by CEF cell while VERO cell the virus can be detected 48 hr pi and maximum titer was 4log₂ (Figure 4).

Comparison between HA titer of virus in tissue culture fluid and cell associated virus

At 120 hr pi we collect tissue culture fluid and cells in separate tube and tested by HA and the result of MDCK cell has slight variation between the 2 collected tubes with 0.5 log high in cell associated virus and in CEF cell has 1 log high in cell associated virus while in Vero cell have 2.5log high in cell associated viruses which related with the intact cell and the virus not released from the cell and we think this viruses were progeny virions not adult one and this is the defect of VERO cell in propagation of avian viruses (Figure 5A-5D).

PCR quantification results

The virus loads in different cell line were measured by absolute quantitative amplification of the target gene M2 in tissue culture infected fluid. Clade 2.2.1.2 viruses were the high amplification copies in MDCK cells 72 hr post infection with concentration 6.5 log₁₀ DNA copies followed by CEF cell 5 log₁₀ and finally VERO cell 4 log₁₀. Whereas other 2 clades of viruses have no significance variation between other which recoded 4.5 log₁₀, 4log₁₀ and 3log₁₀ MDCK, CEF and VERO cell respectively. While H5N8 virus poses concentration 6, 5 and 2log₁₀ in MDCK, CEF and VERO cell respectively so when comparing the virus load in different cell media, it was founded that MDCK cells have the highest concentration of virus load from different virus clades. Overall, the findings imply that a faster virus replication is taking place in MDCK cells than that in CEF and Vero cells (Figure 6A-6D).

Replication kinetics based on TCID₅₀

TCID₅₀ count was performed in different cell line to quantify the virus infectivity and compare growth kinetics between the different clades of circulated viruses in different cell line systems. Clade 2.2.1.2 MDCK growing viruses show the earliest and highest level of multiplicity which can be detected 24 hr pi with concentration 1.5log₁₀ and highest concentration 6.5log₁₀ 96 hr pi with slight variation in another 2 cell lines. The range of infectivity ranged between 4.5 to 5.5log₁₀ at 96 hr pi. Whereas the other clades MDCK growing viruses have infectivity between 3.3 to 5 log₁₀ and first detect in cell culture fluid within 36 hr pi with significance variation ($P<0.05$). The infectivity titer of another 2 clades CEF growing viruses and VERO growing viruses was ranged between 3 to 4 log₁₀ 96 hr pi and viruses can be detected in tissue culture fluid 48 hr pi with significance variation ($P>0.05$). H5N8 MDCK growing virus show the highest level of multiplicity from other 2 cell line with early detection 36 hr pi and the infectivity was 5.8log₁₀ at 96 hr pi while VERO cell growing virus concentration about 2.5log₁₀ and

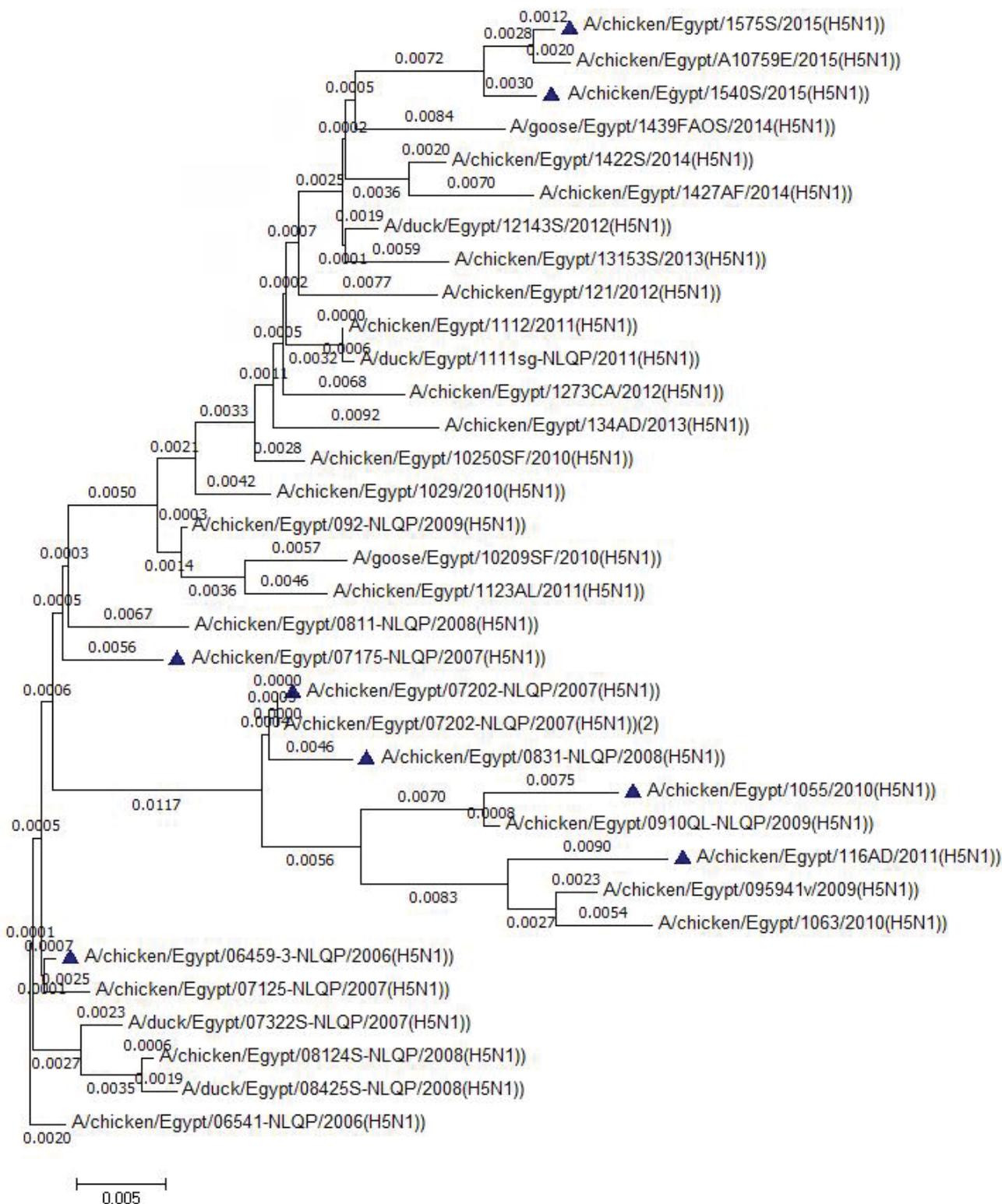


Figure 1: The evolutionary history was inferred using the Neighbor-Joining method [32]. The optimal tree with the sum of branch length=0.20792059 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [34] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7 [31].

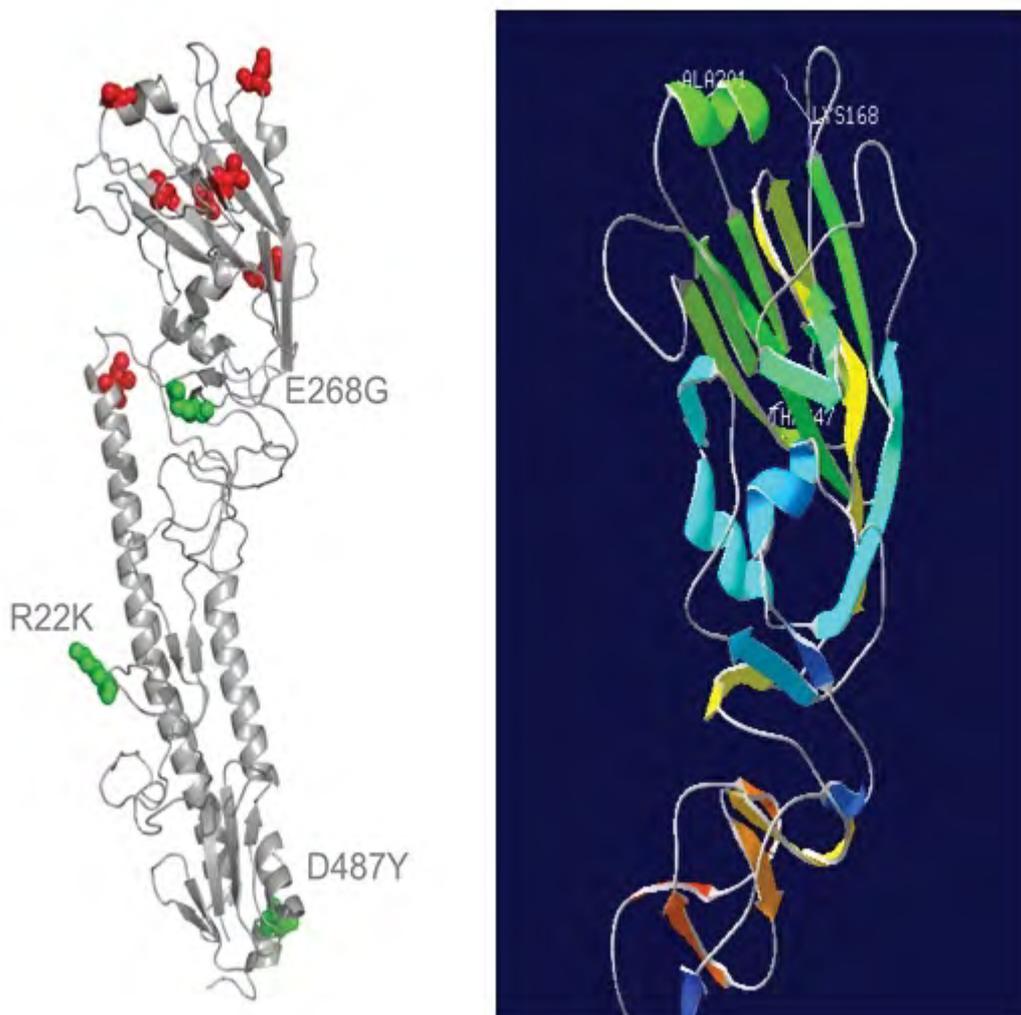


Figure 2A: Three-dimensional structural homology model for the hemagglutinin protein of EG-CA285 created by using the ancestral virus of clade 2.3.4.4b (A/duck/Zhejiang/6D18/2013 [H5N8]) as a template. Amino acids distinguishing the EG-CA285 sequence from the modeling template [17]. **2B:** Three-dimensional structural homology model for the hemagglutinin protein of Clade 2.2.1.2 viruses created by using the ancestral virus of clade 2.2.1 (A/chicken/Egypt/06553/2006[H5N1]) as a template. Amino acids distinguishing the Clade 2.2.1.2 viruses sequence from the modeling template (Figure 3A) CPE Percent of tested viruses in 3 types of cell culture MDCK, CEF and VERO cell explain the difference of growth between viruses in different cell culture through measuring the CPE.

detected 72 hr pi with significance variation ($P>0.05$). Furthermore, all MDCK grown viruses showed higher virus yields than the CEF and VERO cell culture grown virus's (Figure 7).

Antigenic characterization of viruses

Using HI test for antigenic relationship between the different clade of viruses pose clade 2.3.4.4 virus has antigenic relationship with viruses of clade 2.2.1.2 and 2.2.1 while viruses of clade 2.2.1.1 no cross reactivity with serum sample of other clades and vice versa (Table 2). Conversely, no or very few antigenic variation was observed clade 2.2.1.2 and clade 2.2.1 (Table 2).

Discussion

Efficient isolation and propagation of influenza viruses is important in epidemiological surveillance, study of host pathogen interactions, diagnosis and vaccine production. HPAIV subtype H5N1 become an epizootic in Egypt due to the continuous evolution of viruses and

appearance of new clade 2.2.1.2 at the end of 2014 and within the end of 2016 new clade 2.3.4.4 H5N8 viruses isolated from migratory birds in Egypt. This study aim to clarify the possibility of replacement of egg isolation system by tissue culture system and compare 3 different cell cultures to know the ability of each type of cell to cope with continuous changes of viruses and appearance of new viruses in the field in order to get the highest concentration of the virus in the shortest possible time and detection the possibility of transmission to mammalian host. We selected 9 viruses represent clades of virus circulated in Egypt based on phylogenetic analysis and study the growth kinetic of them in 3 cell culture system MDCK, CEF and VERO cell through evaluation of virus load by CPE percent, infectivity and qRT-PCR at designated time points. Many factors effect on ECE and cell culture system for virus isolation such as viral dose, molecular genetic properties of the virus, receptor binding properties of the host cell and some other host related factors lead to successful and efficient propagation [18,19]. New cluster of HPAIV sub type H5N1 has been emerged from clade 2.2.1.2 which

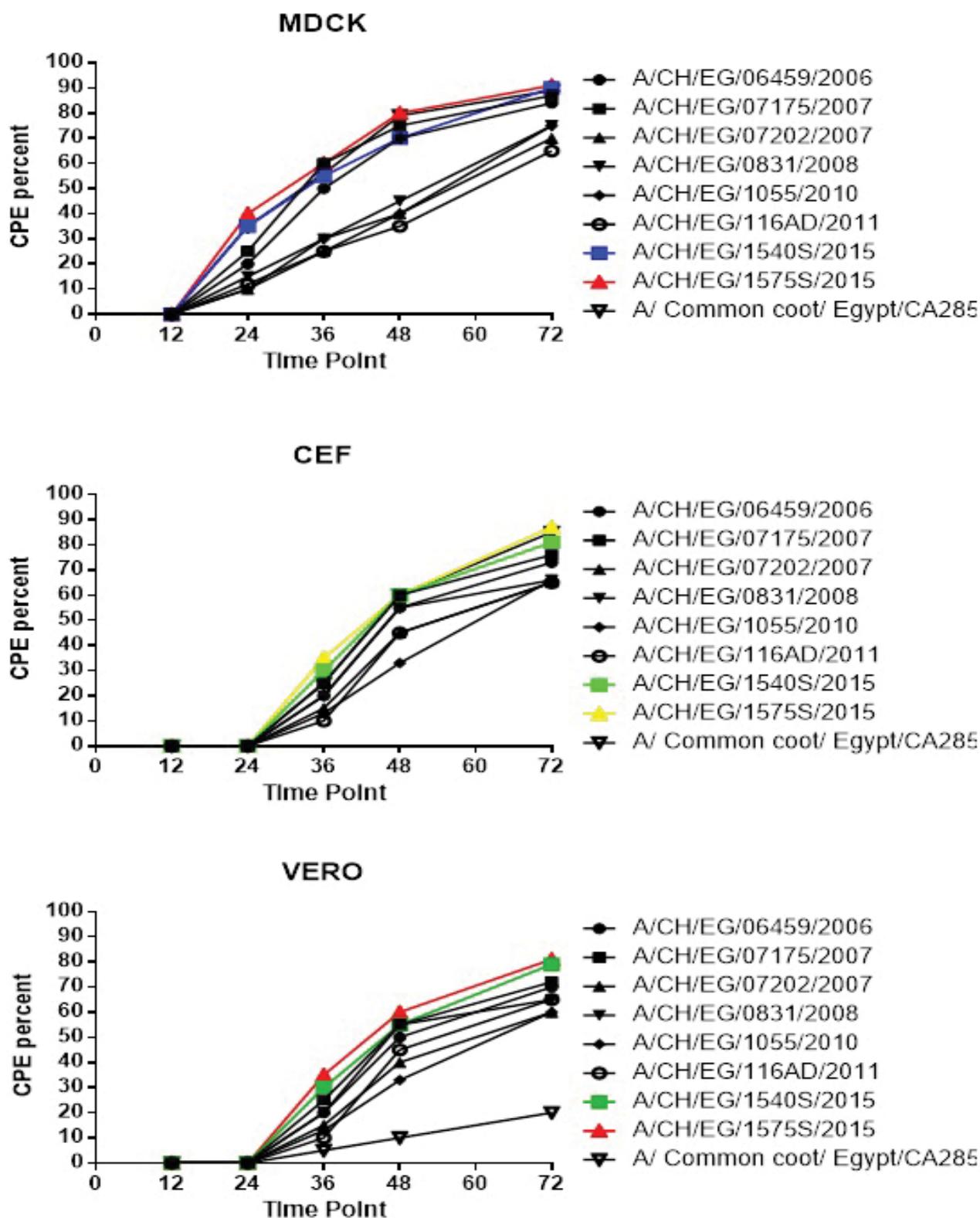


Figure 3A: CPE Percent of tested viruses in 3 types of cell culture MDCK, CEF and VERO cell explain the difference of growth between viruses in different cell culture through measuring the CPE.



B.1: Negative control MDCK 72hr pi



B.2: CPE effect of clade 2.2.1 and 2.2.1.1 viruses on MDCK 72hr pi



B.3: CPE effect of clade 2.2.1.2 and 2.3.4.4 viruses on MDCK 72hr pi



B.4: Negative control CEF 72hr pi



B.5: CPE effect of clade 2.2.1 and 2.2.1.1 viruses on CEF 72hr pi



B.6: CPE effect of clade 2.2.1.2 and 2.3.4.4 viruses on CEF 72hr pi



B.7: Negative control VERO 72hr pi



B.8: CPE effect of clade 2.2.1 and 2.2.1.1 viruses on VERO 72hr pi



B.9: CPE effect of clade 2.2.1.2 and 2.3.4.4 viruses on VERO 72hr pi

Figure 3B: CPE of tested viruses in 3 types of cell culture MDCK, CEF and VERO cell explain the effect of viruses in the cell culture 72 hr post infection showing the difference in CPE between the clades of viruses start from cell lysis to large plaque.

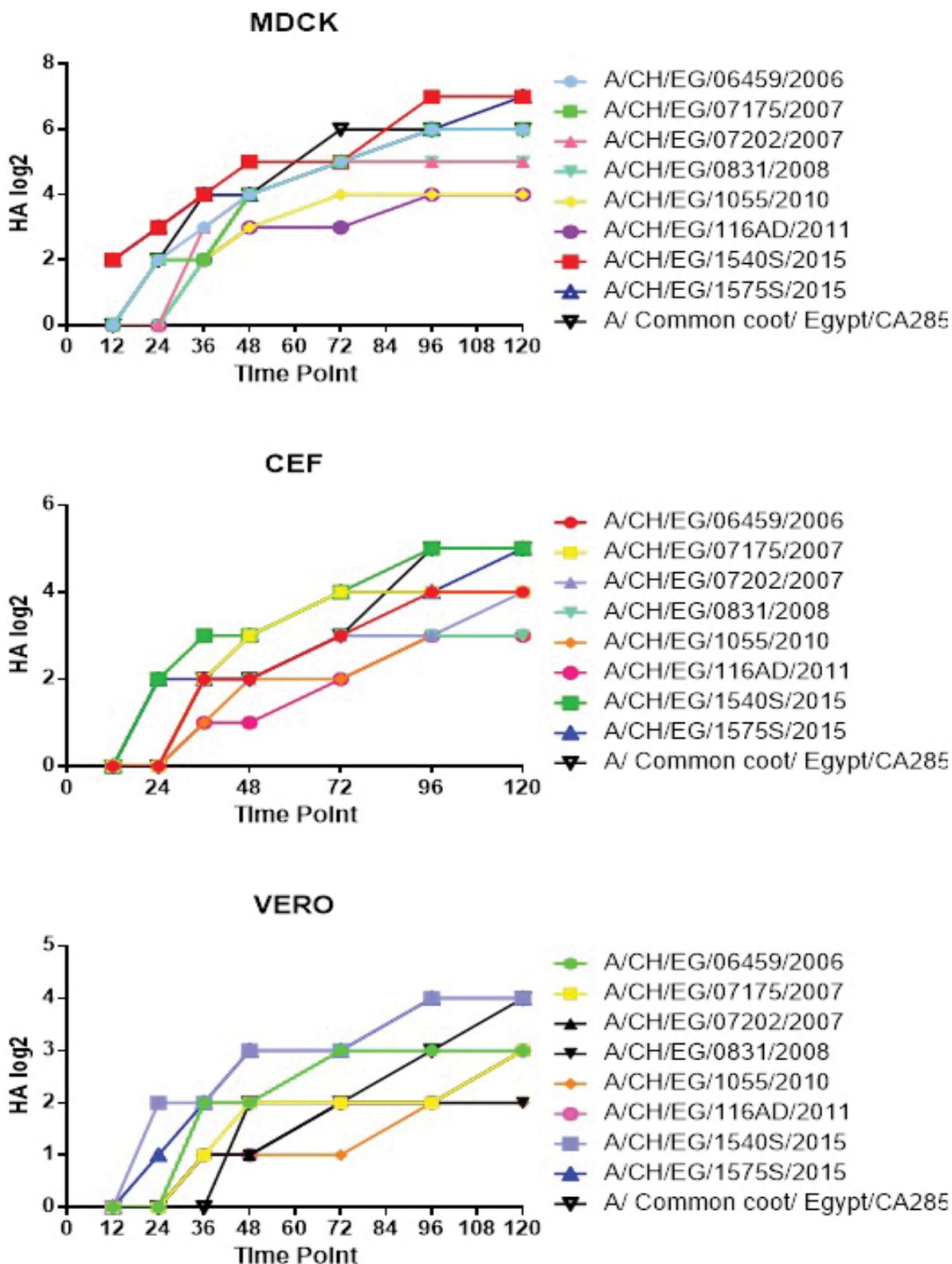


Figure 4: HA result of growing viruses in 3 cell lines and detection of the viruses in tissue culture fluid at different time point explain the difference in growth level between the different viruses.

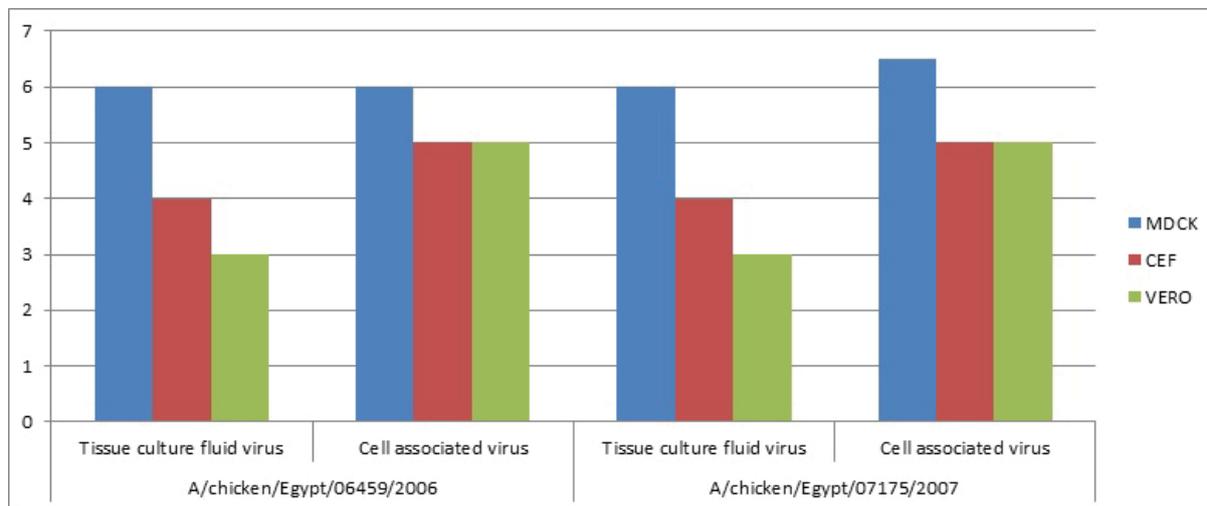


Figure 5A: Result of comparison between HA titer of clade 2.2.1 viruses of cell associated virus and tissue culture fluid virus.

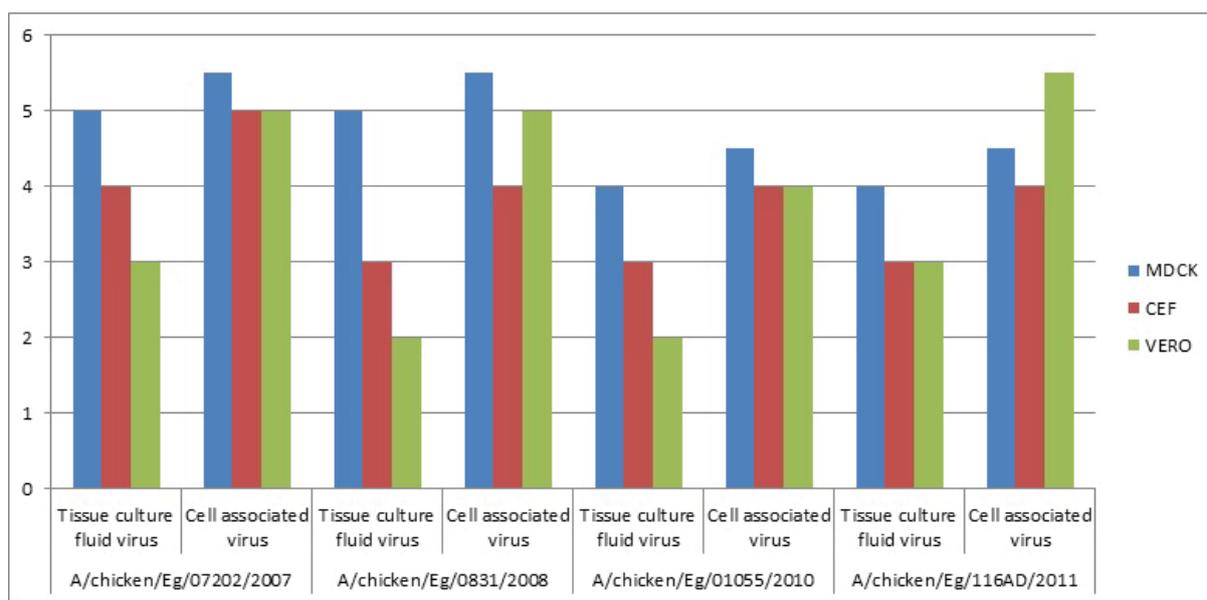


Figure 5B: Result of comparison between HA titer of clade 2.2.1.1 viruses of cell associated virus and tissue culture fluid virus.

Antigen / Antiserum	H5N1	A459/2006	A175/2007	A202/2007	A31/2008	A55/2010	AD6/2011	S40/2015	S75/2015	CA285/2016
H5N1	10	9	7	2	2	3	3	6	6	5
A459/2006	9	10	8	3	3	2	2	7	6	5
A175/2007	8	8	9	3	3	2	2	6	6	4
A202/2007	2	2	3	10	6	6	7	2	2	2
A31/2008	3	2	3	7	9	7	7	3	2	1
A55/2010	3	2	3	7	8	10	9	3	2	1
AD6/2011	3	2	2	7	7	9	10	3	3	2
S40/2015	6	7	6	2	2	3	3	10	8	6
S75/2015	6	7	6	1	2	2	3	8	10	6
CA285/2016	5	5	4	2	1	1	2	6	6	10
SPF serum	1>	1>	1>	1>	1>	1>	1>	1>	1>	1>

Table 2: Antigenic characterisation of highly pathogenic avian influenza A (H5N1 and H5N8) viruses from Egypt based on hemagglutination inhibition assay (log2).

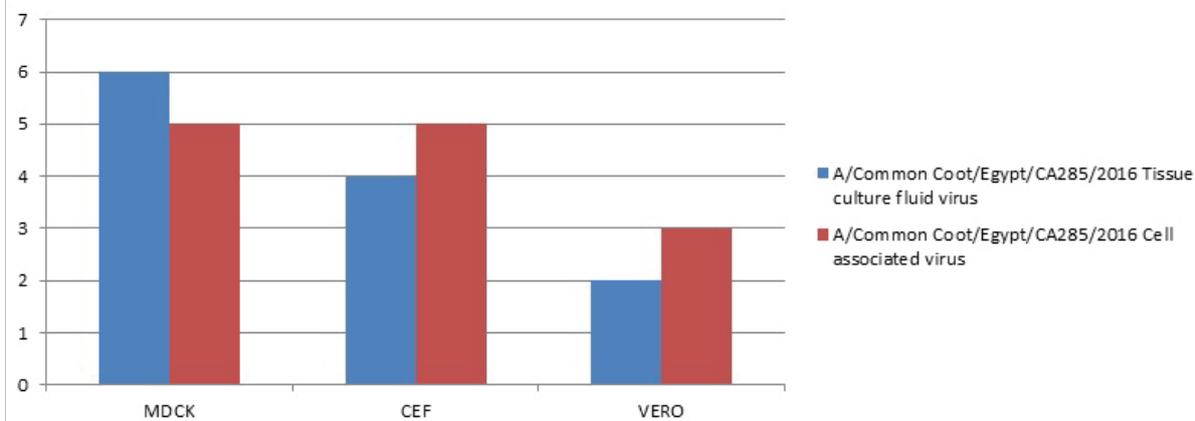


Figure 5C: Result of comparison between HA titer of clade 2.3.4.4 viruses of cell associated virus and tissue culture fluid virus.

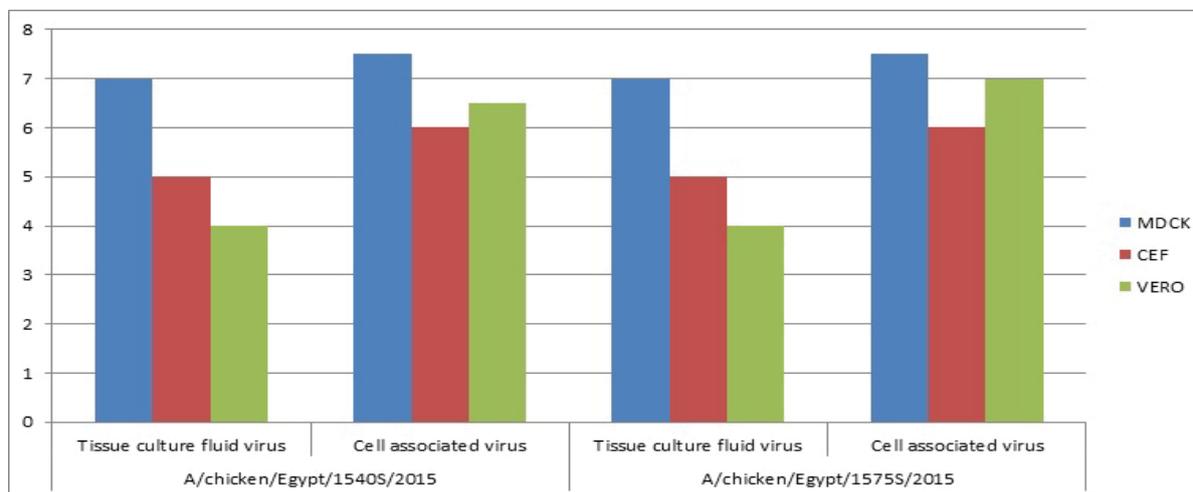


Figure 5D: Result of comparison between HA titer of clade 2.2.1.2 viruses of cell associated virus and tissue culture fluid virus.

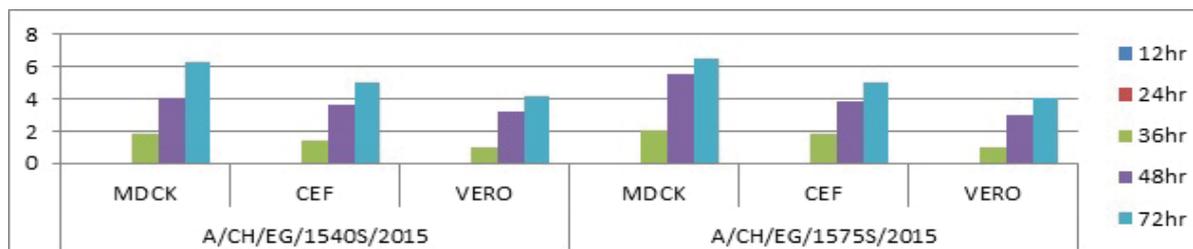


Figure 6A: Explain the replication copies of clade 2.2.1.2 viruses in 3 types of cell culture at different time.

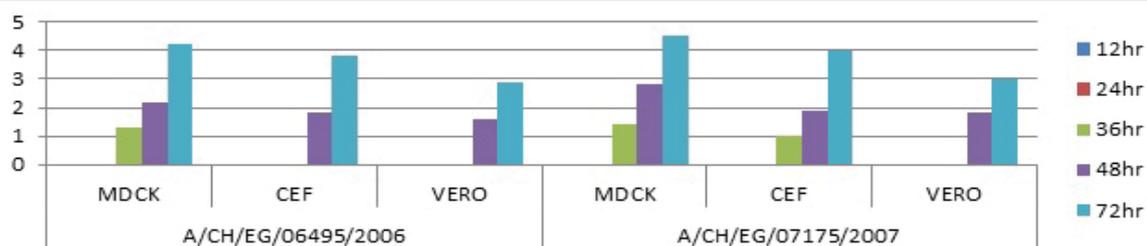


Figure 6B: Explain the replication copies of clade 2.2.1 viruses in 3 types of cell culture at different time.

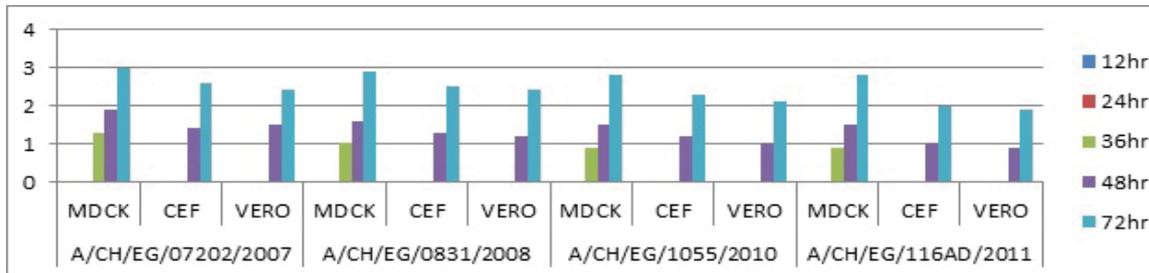


Figure 6C: Explain the replication copies of clade 2.2.1.1 viruses in 3 types of cell culture at different time.

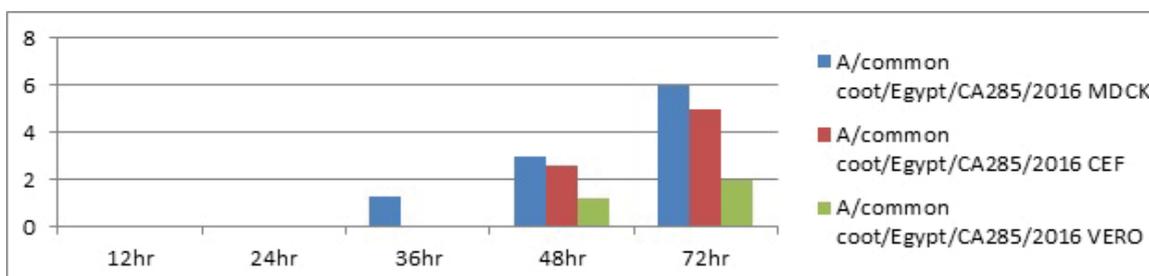


Figure 6D: Explain the replication copies of clade 2.3.4.4 viruses in 3 types of cell culture at different time.

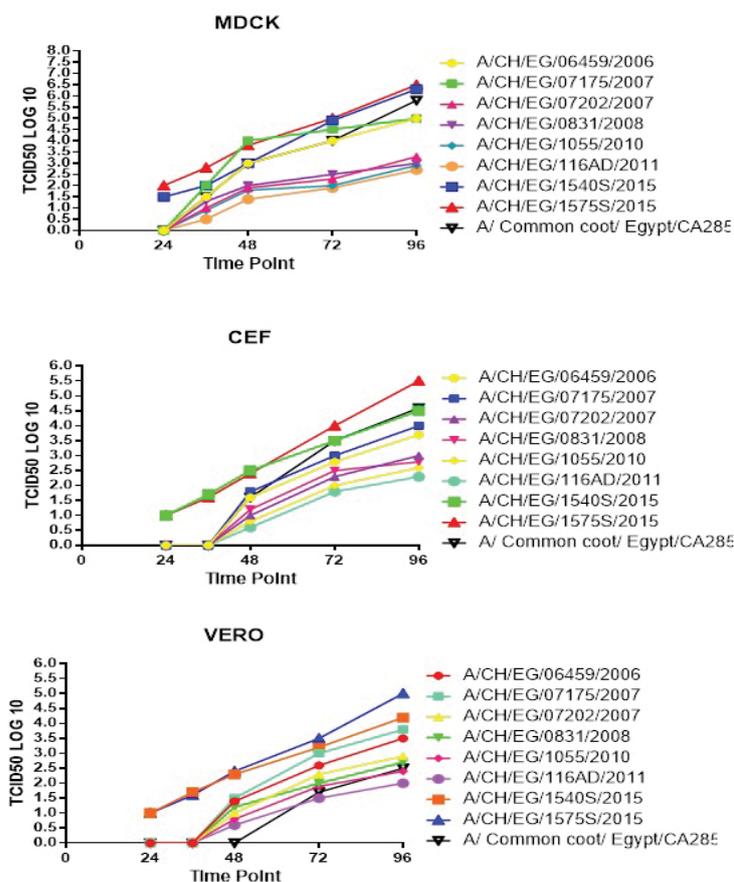


Figure 7: Explain the virus load in different cell line at different time point by calculation of TCID50 at each time point of collected tissue culture fluid.

appear at the end of 2014 and lead to mortalities and sever economic losses and this clade has been descended from clade 2.2.1 HPAI viruses which have been circulated in Egypt from 2006 [20]. The recent evolution of new cluster isn't the first one in Egyptian viruses but it has already developed another evolution in viruses in late 2007 and emerged of 2.2.1.1 which circulated in vaccinated chicken and expanded of escape mutant strain and decline and disappear at 2011 [3]. In late 2016 and during surveillance conducted in birds especially migratory birds at north Egypt 2 cases of avian influenza viruses were isolated from common coot and confirmed HPAIV clade 2.3.4.4 as 1st introduction to Egypt, documenting its introduction into Africa through migratory birds. Pathogenicity of AIV related with the presence of multiple basic amino acids at their cleavage sites and HPAIVs are currently restricted to viruses of H5 and H7 HA subtypes [21]. The replication of influenza virus in a host cell is a polygenic process depending on the host cell endocytic pathways for entry and transfer of viral genome as well as activation of host cell signaling [22]. Efficient replication of avian influenza viruses in host was related with the type of receptors present on the surface of the cells and we can see that in MDCK cell culture which can support replication of both human and avian influenza viruses with high efficiency because both a-2,6- and a-2,3-linked sialic acid receptors are exposed on the MDCK cells [23]. Due to its high susceptibility to various influenza viruses the MDCK cell line remains the most widely used cell line in influenza virus research. Using of MDCK and VERO cell in the study because they are recognized widely and are recommended by the World Health Organization (WHO) for the propagation of various influenza viruses. Firstly MDCK cell line supports the propagation of highly pathogenic avian influenza viruses with the highest load in comparison with CEF or VERO cell culture. The replication ability of the eight H5N1 virus strains and H5N8 virus were assessed separately in 3 systems of tissue culture. The replication ability has been evaluated by the CPE percent, HA test, quantitative PCR and TCID50. MDCK grown viruses exhibited 2-3 log higher than other cell culture grown viruses, however in genome copy number it was 2.5 fold higher than CEF or VERO grown viruses. Although the qRT-PCR method does not take into account the infectious properties of the virus rather can also detect defective virus particles. First and foremost, TCID50 and MOI of the AIV H5N1 virus seed used in the study were determined. It was found that higher TCID50 and MOI values of AIV H5N1 were achieved by propagating the virus in MDCK cells than in CEF and Vero cells. In order to ensure that the AIV H5N1 was provided with sufficient host cells for infection the different cell culture system were infected with 1000 virus particles. MDCK cell began to exhibit CPE within 24 hr pi in comparison to CEF and VERO cells which began to exhibit CPE within 36 hr pi and the maximum CPE percent at 72 hr pi with concentration 91, 86 and 81% in MDCK, CEF and VERO cell respectively for H5N1 but in H5N8 VERO cell poses about 20% CPE 36 hr pi. The percent of CPE reflect the virus load present in the cells so when comparing the virus load produced from each cell culture by estimation of TCID50 we found MDCK grown viruses from different clades of viruses have 1.5log₁₀ higher than other cell grown viruses and early detect in cell culture from 24 hr to 36 hr pi when other cell grown viruses can be detected within 48 hr pi in cell culture, and in comparison between the titer of viruses in remained cells at 120 hr pi in 3 cell line we concluded in MDCK cells there is no variance between the tissue culture fluid viruses and cell associated viruses and the high variation present in VERO cells with about 3 logs high in cell associated virus more than tissue culture fluid one at the 120 hr pi and the cell associated viruses may be brogny virions not adult one and need to multiple passages on cell to be adult and take spherical form of viruses and be able for

infection and the passaging of AIV in Vero cells impair stability of the viruses as discussed before. The similar phenomenon was also observed in a number of previous studies [19]. The scenario of selection of cell culture media in the study due to the accumulated infectious particles could not carry out further infection because more than 90% of the infected cells had died. In view of this, the virus load in cell culture media has slight variation with cell viruses about 0.5log difference as mentioned before [12]. Clade 2.2.1.2 viruses pose the highest replicative viruses in 3 types of cell culture system with early onset of detection of virus particles in cell culture fluid ranged from 12 hr to 36 hr pi with maximum detection within 96 hr pi followed by clade 2.2.1 viruses and finally clade 2.2.1.1 viruses while clade 2.3.4.4 virus H5N8 was highly invasiveness in MDCK cell line and had limited tropism in VERO cell in comparison with H5N1 clade 2.2.1.2 viruses. Thus, the HPAI V H5N8 virus has strong a 2,3-SA receptor-specificity but can also recognize a2,6-SA receptors represent the its lower replication in human cell as discussed before [24,25]. This result has been directed us to possibility of relationship with genetic substitution of the viruses. As discussed in previous study on Egyptian viruses Acquired mutation in HA protein of clade 2.2.1.2 viruses F537S in addition to D94N, T156A, K189R and P235S, mutations which are associated with Improved virus propagation in mammalian cells and increase ability of binding to SA α 2, 6-Gal, the human type of influenza virus receptors [20]. Sequence analysis of clade 2.3.4.4 H5N8 viruses revealed presence of 3 amino acid assignment differences in the HA protein from recent isolate of this viruses in Russia. Clade 2.2.1 viruses have acquired many human-adaptive mutations that can facilitate AI virus replication and transmission in mammals. Among these mutations, we previously reported that the Q192H and I287L/I151T mutations increased the binding affinity of HA for A2, 6 Sialic [26]. Clade 2.2.1.1 viruses were less in propagation in cell line and have less biological fitness in comparison with other viruses clades may be related with the disappearance of this viruses from the field. Antigenic study result can directed us to protection percent between H5N8 viruses and H5N1 viruses which my lead to masking the infection of H5N8 in domesticated poultry and my lead to reassortment between this viruses and enzootic circulated viruses in the field like H5N1 or H9N2 viruses as present in different area. In conclusion cell culture system were suitable for propagation of Egyptian HPAIV either H5N1 or H5N8 and MDCK cell line was the superior type of cell culture system in isolation of viruses and least affected with continues virus evolution in Egypt which confirm the Egyptian viruses still prefer to bound with avian receptors more than mammalian type and also we can use it for replacing of egg isolation system [27-34].

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References

1. Group WOFHNEW (2009) Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. *Influenza Other Respir Viruses* 3: 59-62.
2. Smith GJ, Donis RO (2015) World Health Organization/World Organisation for Animal HF, Agriculture Organization HEWG. Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. *Influenza Other Respir Viruses* 9: 271-6.
3. Arafa A, Suarez D, Kholosy SG, Hassan MK, Nasef S, et al. (2012) Evolution of highly pathogenic avian influenza H5N1 viruses in Egypt indicating progressive adaptation. *Arch Virol* 157: 1931-1947.
4. Arafa A, Masry IE, Kholosy S, Mohammed KH, Dauphin G et al. (2016) Phylodynamics of avian influenza clade 2.2.1 H5N1 viruses in Egypt. *Virology Journal* 13: 49.

5. Tseng YF, Hu AYC, Huang ML, Yeh WZ, Weng TC, et al. (2011) Adaptation of High-Growth Influenza H5N1 Vaccine Virus in Vero Cells: Implications for Pandemic Preparedness. *PLoS ONE* 6: e24057.
6. Pierre-Louis H, Lorin V, Jouvion G, DaCosta B, Escriou N (2015) Addition of N-glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity. *Virology* 486: 134-145.
7. Abdoli A, Soleimanjahi H, Tavassoti Kheiri M, Jamali A, Jamaati A, (2013) Determining influenza virus shedding at different time points in Madin-Darby canine kidney cell line. *Cell Journal* 15: 130-135.
8. Genzel Y, Dietzsch C, Rapp E, Schwarzer J, Reichl U (2010) MDCK and Vero cells for influenza virus vaccine production: A one-to-one comparison up to lab-scale bioreactor cultivation. *Applied Microbiology and Biotechnology* 88: 461-475.
9. Youil R, Su Q, Toner TJ, Szymkowiak C, Kwan WS, et al. (2004) Comparative study of influenza virus replication in Vero and MDCK cell lines. *J Virol Methods* 120: 23-31.
10. Imai M, Kawaoka Y (2012) The role of receptor binding specificity in interspecies transmission of influenza viruses. *Curr Opin Virol* 2: 160-167.
11. Yamaji R, Yamada S, Le MQ, Ito M, Sakai-Tagawa Y, et al. (2015) Mammalian adaptive mutations of the PA protein of highly pathogenic avian H5N1 influenza virus. *J Virol* 89: 4117-4125.
12. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. *The American Journal of Epidemiology* 27: 493-497.
13. Abdoli A, Soleimanjahi H, Jamali A, Mehrbod P, Gholami S, et al. (2016) Comparison between MDCK and MDCK-SIAT1 cell lines as preferred host for cell culture-based influenza vaccine production. *Biotechnol Lett* 38: 941-948.
14. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 40: 3256-3260.
15. OIE (2015) Avian influenza. Chapter 2.3.4 (Last Accessed August 15, 2015).
16. Katoh K, Standley DM (2014) Iterative refinement and additional methods. *Methods Mol Biol*, pp: 131-146.
17. Selim AA, Erfan AM, Hagag N, Zanaty A, Samir AH, et al. (2017) Highly Pathogenic Avian Influenza Virus (H5N8) Clade 2.3.4.4 Infection in Migratory Birds, Egypt. *Emerg Infect Dis* 23: 1048-1051.
18. Lebas ZN, Shahla S, Ashraf M, Mohammad ME, Mehran B, et al. (2013) Replication efficiency of influenza A virus H9N2: a comparative analysis between different origin cell types. *Jundishapur J Microbiol* 6: e8584.
19. Youil R, Su Q, Toner TJ, Szymkowiak C, Kwan WS, et al. (2004) Comparative study of influenza virus replication in Vero and MDCK cell lines. *J Virol Methods* 120: 23-31.
20. Arafa AS, Naguib MM, Luttermann C, Selim AA, Kilany WH, et al. (2015) Emergence of a novel cluster of influenza A (H5N1) virus clade 2.2.1.2 with putative human health impact in Egypt, 2014/15. *Euro Surveill* 20: 2-8.
21. Nao N, Kajihara M, Manzoor R, Maruyama J, Yoshida R, et al. (2015) A Single Amino Acid in the M1 Protein Responsible for the Different Pathogenic Potentials of H5N1 Highly Pathogenic Avian Influenza Virus Strains. *PLoS ONE* 10: e0137989.
22. Zhang H (2009) Tissue and host tropism of influenza viruses: importance of quantitative analysis. *Sci China C Life Sci* 52: 1101-1110.
23. Hatakeyama S, Sakai-Tagawa Y, Kiso M, Goto H, Kawakami C, et al. (2005) Enhanced expression of an α 2,6-linked sialic acid on MDCK cells improves isolation of human influenza viruses and evaluation of their sensitivity to a neuraminidase inhibitor. *J Clin Microbiol* 43: 4139-4146.
24. Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D (1994) Apoptosis: A mechanism of cell killing by influenza A and B viruses. *Journal of Virology* 68: 3667-3673.
25. Kaplan BS, Russier M, Jeevan T, Marathe B, Govorkova EA, et al. (2016) Novel highly pathogenic avian A(H5N2) and A(H5N8) influenza viruses of clade 2.3.4.4 from North America have limited capacity for replication and transmission in mammals. *mSphere* 1: e00003-16.
26. Watanabe Y, Ibrahim MS, Ellakany HF, Kawashita N, Mizuike R, et al. (2011) Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. *PLoS Pathog* 7: e1002068.
27. Aly MM, Arafa A, Hassan MK (2008) Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. *Avian Dis* 52: 269-277.
28. Govorkova EA, Kaverin NV, Gubareva LV, Meignier B, Webster RG (1995) Replication of influenza A viruses in a green monkey kidney continuous cell line (Vero). *The Journal of Infectious Diseases* 172: 250-253.
29. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 146: 2275-2289.
30. Nakowitsch S, Waltenberger AM, Wressnigg N, Ferstl N, Triendl A, et al. (2014) Egg- or cell culture-derived hemagglutinin mutations impair virus stability and antigen content of inactivated influenza vaccines. *Biotechnol J* 9: 405-414.
31. Kumar S, Stecher G, Tamura K (2016) MEGA 7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874.
32. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.
33. Yoon SW, Kayali G, Ali MA, Webster RG, Webby RJ, et al. (2013) A Single Amino Acid at the Hemagglutinin Cleavage Site Contributes to the Pathogenicity but Not the Transmission of Egyptian Highly Pathogenic H5N1 Influenza Virus in Chickens. *Journal of Virology*, pp: 4786-4788.
34. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* 101: 11030-11035.