Vet. Med. J., Giza. Vol. 59, No. 3 (2011): 47 - 63

Tissue culture propagation of highly pathogenic avian influenza H5N1 virus

Soliman, Y. A.; Afaf, A. Khedr; Nermeen, M. Elsaid; Abdel Baky, M. H. and Elham, A. Ebiary.

Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo.

Received: 19/04/2011
Accepted: 08/05/2011

SUMMARY

Avian influenza virus usually refers to influenza A viruses found chiefly in birds, but infections can occur in humans. The risk is generally low to most people, because the viruses do not usually infect humans. However, confirmed cases of human infection have been reported since 1997 by H5N1 subtype. In the current study, a highly pathogenic avian influenza subtype H5N1 was isolated and confirmed by PCR and sequencing. Sequence analysis revealed a high degree of identity with other published sequences of H5N1 isolates from Egypt. The multiple basic amino acid sequence of the isolated virus was similar to the highly pathogenic strains of H5N1 (G-E/R-R-R/K-K-R.) and to other viruses isolated in Egypt in the previous years specially in the presence of arginine amino acid in the cleavage site motive of the hemagglutinin protein. The isolated virus which designed as A/chicken/1000CLEVB/2010 was propagated on chicken embryo fibroblast tissue culture. The virus titer was rapidly increasing from 4.8 log_{10} /ml in the 1st passage to 8.5 log_{10} /ml by the 3rd one. The HA properties of the virus was regained by treatment with 0.5µg of trypsin /10 min which open the gates for the production of inactivated tissue culture propagated vaccine

INTRODUCTION

Influenza viruses are enveloped, pleomorphic and negative sense segmented RNA viruses belonging to the family Orthomyxoviridae. These viruses have been isolated from a wide range of hosts including humans, pigs, birds, horses and sea mammals, and are classified as types A, B and C based on the antigenic differences in two of their gene products, nucleoprotein (NP) and matrix (M1).

Avian influenza is caused by type A influenza virus, which is further classified into subtypes on the basis of two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Accordingly, 16 HA (HA1–HA16) and nine NA (NA1–NA9) subtypes have been identified (Fouchier *et al.*, 2005)

Highly pathogenic avian influenza H5N1 (HPAIV) was first identified in 1996 as the causative agent of the geese outbreak in the Guangdong Province, China (Xu *et al.*, 1999); in 1997, the same subtype caused outbreaks in chickens, with 18 human infections reported in Hong Kong.

In mid-February 2006, H5N1 infection was reported in Egypt among domestic poultry in more than 15 governorates (Bahgat *et al.*, 2009), resulting in severe losses for the poultry industry. The Egyptian government initiated a poultry stamping out program that appeared to limit the 2006 outbreak; however, H5N1 viruses reemerged in a series of avian epidemics in several governorates. From 2006 till date, there have been 1084 outbreaks of H5N1 among poultry in Egypt (OIE, 2008). According to data published by the WHO, of the 129 cases confirmed to date in Egypt (March 2011), 43 have been fatal. These infected humans were in close contact mainly with domestic poultry in rural areas (http://www.who.int/csr/don/2011_03_10/en).

Hemaglutinin gene product is predominantly responsible for pathogenicity of AIV. This gene is about 1778 bp long with an open reading frame of 1704 bases (568 amino acid residues). Based on the pathogenicity of AIVs in domestic poultry, these are classified into two pathotypes. highly pathogenic avian influenza (HPAI) and non-highly pathogenic avian influenza (nHAPI) including mild pathogenic, low pathogenic and non-pathogenic AIV (Swayne *et al.*, 1997). Cleavage of HA molecule (HA_0), by host cell proteases, into HA_1 and HA_2 subunits is essential for infectivity, and AIVs with high and low levels of pathogenicity differ in their cleavage sequence

AI vaccination in Egypt has been the main tool used over the past years to control the H5N1 HPAI epidemic. The focus on vaccination appears to have distracted attention away from other essential/critical procedures and disease control measures such as surveillance, bio-security, outbreak investigation, and disease management interventions. Indeed, more than 80% (24 million USD) of the available budget for HPAI control has been devoted to vaccination since the programme was launched (GOVS, 2009).

The commercial vaccines available in Egypt for H5N1 AIVs include two oil-emulsion formulations: one manufactured from north

American and European strain of AI subtype H5N2 virus and the other from a reassortant H5N1 virus carrying the H5 gene from Egyptian isolate of H5N1. The main concerning issue in these vaccines is that they were not produced from local Egyptian field isolate. For production of a highly efficient vaccine, the strains used to produce the vaccine must be sufficiently closely related to the circulating strains to ensure the induction of effective protective immunity against infection (Hoffmann *et al.*, 2002). Sequence analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of selected Egyptian isolates from early 2006 to 2008 identified distinct genetic markers in both HA and NA genes and suggests grouping Egyptian isolates into two major HA isolate sublineages from 2006 to 2008 and into three smaller, emergent subgroups. The NA phylogenetic and sequence analysis showed a similar pattern, except that two of the emergent groups from the HA phylogenetic tree clustered together (Arafa *et al.*, 2010)

Production of inactivated vaccine is hampered by the low titer of virus when propagated on SPF eggs due to the high pathogenicity of the virus that kills the embryo within 24 hours.

This study was undertaken to investigate the property of a field isolate of highly pathogenic avian influenza H5N1virus from Egyptian farms during early 2010 and to propagate this isolate on SPF embryonated chicken fibroblast (CEF) as a tool for preparation of tissue culture propagated vaccine or antigen preparation

MATERIALS AND METHODS

Virus isolation:

Tracheal swabs were taken from recently dead chickens from poultry farms showing severe clinical signs of influenza infection with mortality rate exceeding 70% , the swabs were soaked in phosphate buffered saline (pH7.2) containing 100 U/ml penicillin and 100µg/ml streptomycin. The solution was then centrifuged at 5000rpm/10min/4 °C, the supernatant was then collected and filtrated using 0.22µ syringe millipore filter before inoculation in 9 days old SPF chicken eggs via the Allantoic route. The eggs were incubated at 37°C in a humid champers till embryo death (usually within 24 hours) as confirmed by candling.

Oligonucleotides, cDNA synthesis, and RT-PCR amplification

Viral RNA was extracted from the clarified Allantoic fluid and from virus propagated on CEF cell culture by total RNA purification kit (BioFlux, Canada), according to the manufacturer's instructions. The first strand

cDNA was synthesized with the fermantase H minus kit according to the manufacture instructions, the PCR amplification was done with green master mix (fermantase) using the following primers that target the HA cleavage site, Sense primer, 5'–CCT-CCA-GAA-TAT-GCG-TAG -3' and the Antisense primer. 5' - TAC-CAA- CCG- TCT- ACC- ATG- CCG -3'. The amplicons were electrophoresed on a 1% agarose.

Sequencing:

The complete nucleotide sequences of the amplicon was performed by ABI prism 3310 gene analyzer. For preparation of the gene for sequencing, the PCR product was separated on 0.7 % low melting agarose and electrophoresed on low voltage (20volt) at 4°C. The bands were sliced off and purified with the Biospin agarose purification kit (BioFlux, Canada) as described by the manufacturer. Briefly, the gel slices were melted at 60°C for 5 min, mixed with 500 µl of gel extraction buffer and placed on the biospin column provided with the kit, centrifuged at 4000xg/2min/4°C and washed twice with the washing solution. Finally the amplicon was eluted in 50 µl of the elution buffer and the concentration was adjusted to 0.1µg/µl and stored at -20 °C till used.

Analysis:

The sequence analyses was performed using CLC Sequence Viewer Version 6.3 Windows 7.6.1 platform developed by CLC bio A/S and FastPCR professional 6.1.9 beta 3 package.

Virus propagation on Embryonated chicken fibroblasts:

Chicken embryo fibroblasts were cultured; the culture medium, is composed of MEM supplemented with 10 % fetal calf serum (*Lonza Walkersville, Inc*). CEF were infected as secondary cultures When the monolayers were between 90 to 95% confluent with the with a low multiplicity of infection (MOI) (0.1) in a 100-µl inoculums (Catherine *et al*., 2006) of the SPF egg propagated H5N1 virus. Culture showed CPE in the form of rounding and detaching were freezed and thawed twice and clarified by centrifugation at 5000rpm/15 min/4°C. The supernatant was used for further propagation on SPF embryonated chicken eggs or re-inoculation of the CEF, PCR exterminate and HA.

Determination of the $TCID_{50}$

Serial ten-fold dilutions of the 3rd passage of the H5N1 virus in the growth medium were prepared. A 200-µl amount of each dilution was placed in each of 8 culture wells with CEF cells and allowed to absorb for 30 min at 37°C, then 2-ml MEM without FCS was added and the cultures were incubated at 37°C until finally assessed at 5 days. The cultures were observed daily and both positive

and negative results were recorded. The $TCID_{50}$ was calculated from the scores by the method of Reed and Muench (1938).

Hemaglutination test:

Virus clarified from the Allantoic fluid or propagated on culture of CEF were used in the HA assay (Killian 2008). the CEF propagated virus was used pre treated with trypsin 0.5µg/ml for different time durations , and freshly prepared 1% washed erythrocytes of SPF chicken blood was used to conduct the HA assay. Virus titration was then calculated using the reed and munch1938.

Hemaglutination inhibition test:

It was done according to Katz *et al.*, 2009 and Potter and Oxford 1979. Monospecific antisera against H5N1 (prepared from A/ Gs/Gd /1/96 H5N1) was used to determine the Hemaglutination inhibition of 4 HA units of egg propagated virus . Monospecific antisera against other Hemaglutinating agent as Newcastle virus (standard newcastle Anti-serum obtained from CVL weybridge) and egg drop syndrome were used to evaluate the purity of the isolate.

RESULTS

Virus isolation and characterization:

The clinical signs of AI infection in poultry farms were characteristics ranging from drop in egg production, hemorrhagic spots on the leg shafts, swollen cyanotic combs. In the P/M there was severe muscular hemorrhage, with petechial hemorrhagic on the peritoneum. The mortality rate begun in drastic increasing fusion which reached to 70% within 3 days. Samples from the recently died birds were transported on ice to the lab were individual swabs were taken from the trachea and inoculated in the SPF embryonated chicken eggs. Those showed embryo mortalities within 24 h were chilled and the chorioallantoic fluid was harvested and virus characterization was done by RT-PCR using specific primers directed towards the H5 cleavage site. As shown in Fig 1 , clear visible band with molecular size of ~ 315 bp having high intensity was seen from the inoculated SPF eggs either with the original sample (lane 1) or virus propagated on TC then returned to the SPF eggs (lane 2). It was noticed that virus propagated on TC gave bands with the same molecular size (lane 6 and 7) but differ in the intensity which is roughly indicated the low concentration of the virus in the starting samples for RNA extraction.

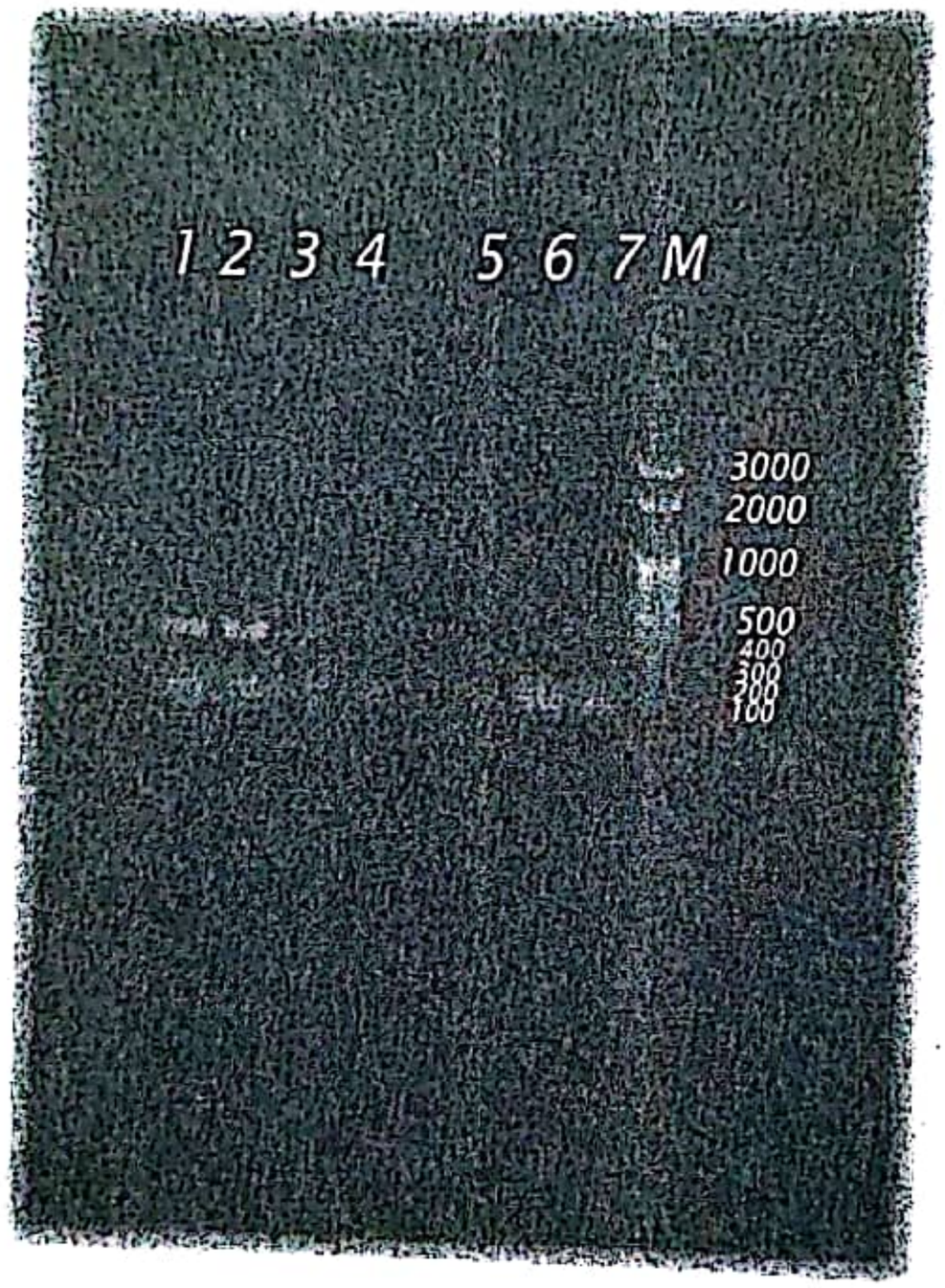

Fig 1: the PCR amplification of cleavage site of H5 gene of H5N1 virus purified from the Allantoic fluid (lane 1) TC propagated and then egg propagated (lane 2) , TC propagated virus of the 1st, 2nd, 3rd passage (lane 3, 6,7) . lanes 4 and 5 negative control from the uninfected Allantoic fluid or uninfected CF .

Sequencing analysis:

Sequence alignment was performed using CLC sequence viewer software between the 264 bp of the amplicon under study (excluding the primer sequence) with the corresponding sequence of H5 gene of 2009 Egyptian isolated http://www.ncbi.nlm.nih.gov/nuccore/260104174?from=1&to=1707&report=gbwithparts

Comparison of the amino acid sequence at the cleavage site was performed between the field isolate designated as

(A/chicken/Kgarbia/ch100/2010(H5N1)) and other published sequences (Lee *et al.*, 2005)

BLAST search revealed a great similarity with strains isolated from Egypt during the last 5 years with identity varies between 98%-100% (table 2)

Table (1): Comparison of amino acid sequences of H5 cleavage site of the field isolate and other H5N1 strains

strain	Cleavage site (323–330)
(A/chicken/Kgarbia/ch100/2010(H5N1))	GERRRKKR
(A/chicken/Egypt/09519S-NLQP/2009)	GEGRRKKR
(A/chicken/Egypt/1022L/2010(H5N1))	GERRRKKR
(A/chicken/Egypt/1058sf/2010(H5N1))	GERRRKKR
(A/chicken/Egypt/091317s/2009(H5N1))	GERRRKKR
(A/duck/Egypt/D3Li12/2007(H5N1))	GERRRKKR
(A/duck/Egypt/D2Li234/2007(H5N1))	GERRRKKR
(A/chicken/Egypt/38-2/2008(H5N1))	GERRRKKR
(A/chicken/Egypt/37-1/2008(H5N1))	GERRRKKR
(A/chicken/Egypt/35-3/2008(H5N1))	GERRRKKR
(A/chicken/Egypt/33-1/2008(H5N1))	GERRRKKR
A/chicken/Egypt/492N3-CLEVB/2008(H5N1))	GERRRKKR
(A/chicken/Egypt/490N3-CLEVB/2008(H5N1))	GERRRKKR
(A/chicken/Egypt/488N3-CLEVB/2008(H5N1))	GERRRKKR
(A/chicken/Egypt/0987alb-NLQP/2009(H5N1))	GERRRKKR
HK/483/97	RERRRKKR
GS/437-4/99	RERRRKKR
CK/HK/YU822.2/01	RERRRKKR
DK/HK/821/02	IERRRKKR
DK/China/E319-2/03	RE-RRRKR
CK/Korea/ES/03	RE-KRKKR
Thailand/1(KAN-1)/04	RERRRKKR
Vietnam/1196/04	RERRRKKR

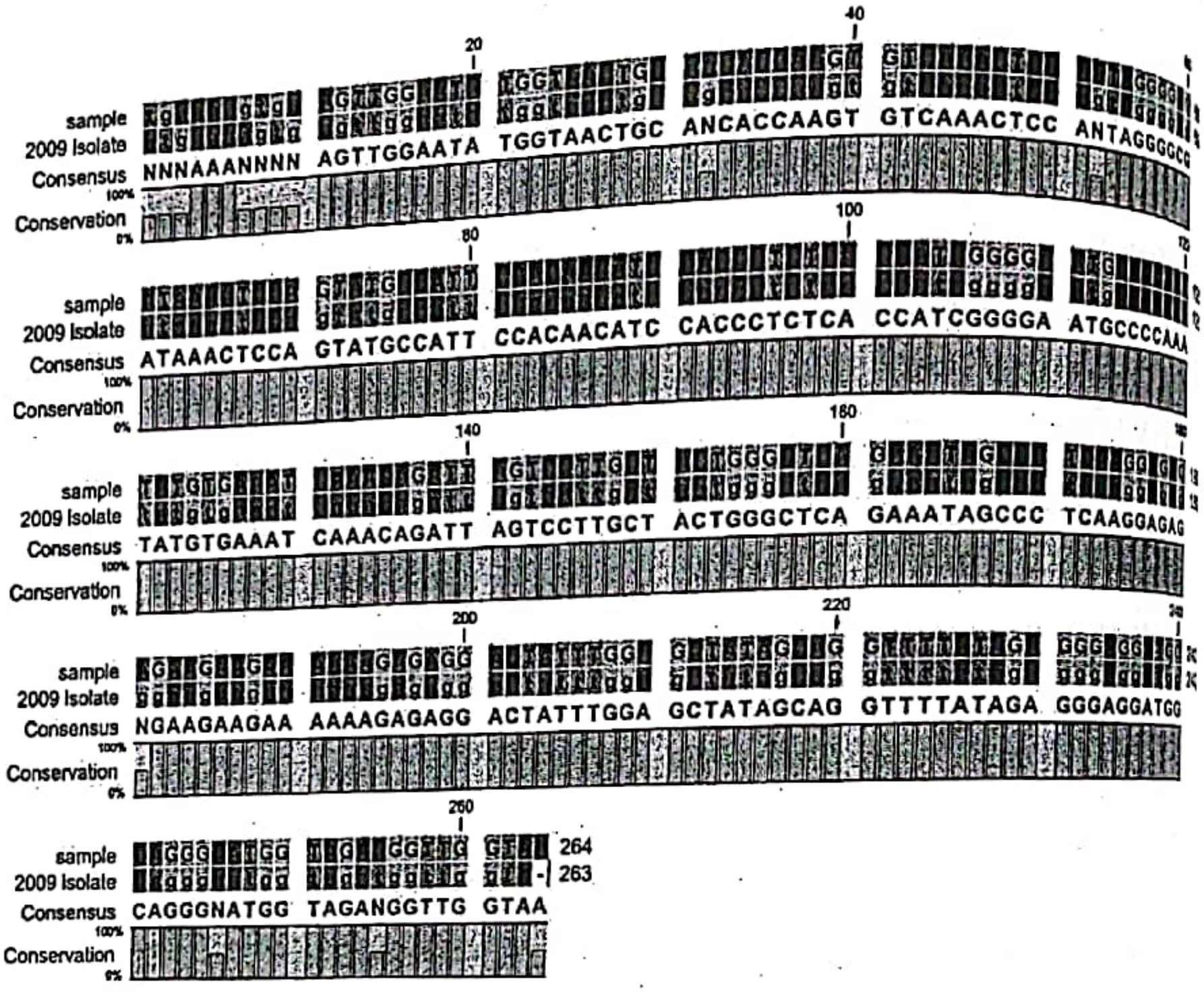

Fig 2: nucleotide sequence alignment between the sequence of the field isolate (A/chicken/Kgarbia/ch100/2010(H5N1)) (sample) and the published sequence of the H5 gene (2009 isolate) in gene bank data base.

Table (2): Sequences producing significant alignments with the filed isolate of avian influenza H5N1 (A/chicken/Kgarbia/ch100/2010(H5N1))

Accession	Description	Total score	Query coverage	E value	Max ident
HQ198295.2	Influenza A virus (A/chicken/Egypt/10159s/2010(H5N1)) segment 4 hemagglutinin (HA) gene, complete cds	486	100%	2e-134	100%
HQ198269.2	Influenza A virus (A/chicken/Egypt/1063/2010(H5N1)) segment 4 hemagglutinin (HA) gene, complete cds	486	100%	2e-134	100%
HQ198251.1	Influenza A virus (A/chicken/Egypt/095723v/2009(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	486	100%	2e-134	100%
HM466695.1	Influenza A virus (A/chicken/Israel/65/2010(H5N1)) segment 4 hemagglutinin (HA) gene, complete cds	481	100%	1e-132	99%
CY062601.1	Influenza A virus (A/chicken/Egypt/18-H/2009(H5N1)) segment 4 sequence	481	100%	1e-132	99%
GU002695.1	Influenza A virus (A/chicken/Egypt/09568S-NLQP/2009(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	475	100%	5e-131	99%
HQ198278.2	Influenza A virus (A/chicken/Egypt/1071g/2010(H5N1)) segment 4 hemagglutinin (HA) gene, complete cds	470	100%	2e-129	98%
AB497019.1	Influenza A virus (A/chicken/Egypt/C1Lu2/2007(H5N1)) HA gene for hemagglutinin, complete cds	470	100%	2e-129	98%
CY061552.1	Influenza A virus (A/chicken/Egypt/1/2008(H5N1)) segment 4 sequence	470	100%	2e-129	98%
GU811716.1	Influenza A virus (A/duck/Egypt/08561S-NLQP/2008(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	470	100%	2e-129	98%
GU811712.1	Influenza A virus (A/chicken/Egypt/0882-NLQP/2008(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	470	100%	2e-129	98%
CY055191.1	Influenza A virus (A/turkey/Egypt/7/2007(H5N1)) segment 4 sequence	470	100%	2e-129	98%
FJ472343.1	Influenza A virus (A/chicken/Qalubia/1/2006(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	470	100%	2e-129	98%
FJ226061.1	Influenza A virus (A/Egypt/3300-NAMRU3/2008(H5N1)) segment 4 hemagglutinin (HA) gene, partial cds	470	100%	2e-129	98%
EU719116.1	Influenza A virus (A/egret/Egypt/1162-NAMRU3/2006(H5N1)) hemagglutinin (HA) gene, partial cds	470	100%	2e-129	98%

Tissue culture propagation of H5N1 isolate.

The virus confirmed to be H5N1 with the PCR and sequencing analysis was inoculated in CEF cultured cells. The cytopathic effect (CPE) started to appear at about 48h post infection reaching its maximum at 5 days post infection (fig 3). Further two serial passage of the virus did not elicit any difference in the shape and

duration of the CPE. $TCID_{50}$ of the virus scored 4.8 log_{10} /ml in the first 2 passages but was abruptly increased to 8.5 log_{10} /ml by the 3rd passage.

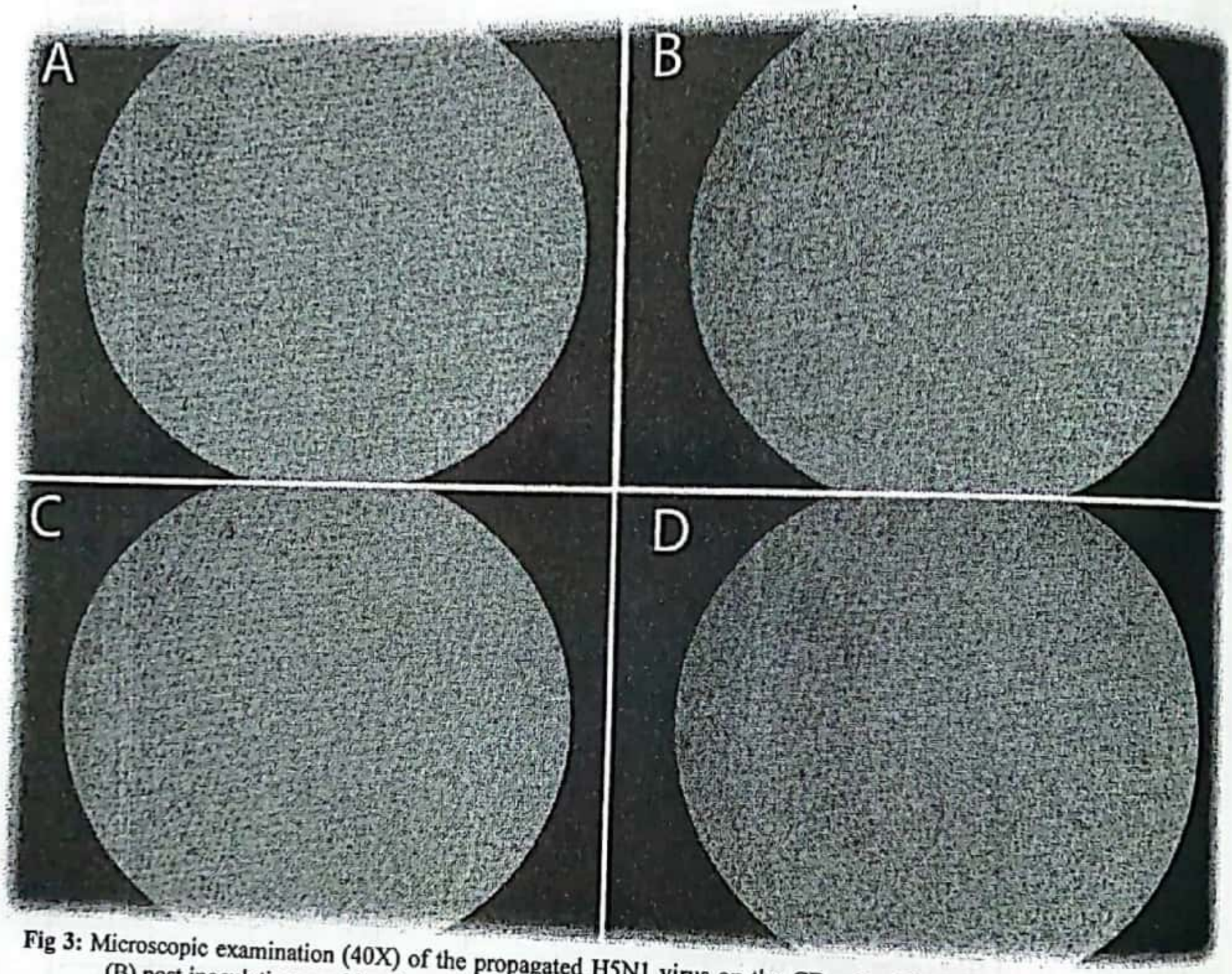

Fig 3: Microscopic examination (40X) of the propagated H5N1 virus on the CF cells. The CPE begun to appear 2 days (B) post inoculation reaching its maximum after 5 days (D). The CPE was in the form of rounding and detaching of the cells.

Hemaglutination test.

Table (2) shows the results of HA test of SPF propagated Vs tissue culture propagated virus. Virus propagated in eggs showed increased in the HA titer by serial passage. concerning the tissue culture propagated virus , no evidence of HA activity were seen at any dilutions with non treated virus where the trypsin treated virus showed HA titer of 5 in the first passage increased to 7 with the 3rd passage. Virus propagated in tissue culture followed by egg propagation gave higher HA titer than trypsin treated TC propagated virus.

Table (3) Hemaglutination titer (expressed in Log 2) of the H5N1 virus propagated either on SPF embryonated chicken eggs and/ or CF tissue culture.

virus	Passage #		
	1	2	3
Embryonated chicken egg propagated virus	6	7	12
CEF propagated and trypsin treated virus	4	5.5	8
CEF propagated virus	0	0	0
CEF then Embryonated chicken egg propagated virus	5	6	9

Hemaglutination inhibition test.

Monospecific antisera against AIV, subtype H5N1 strain gave HI titer of 12 by using 4HAU of the 3rd passage of the isolate, while using monospecific antisera against NDV and egg drop syndrome gave no detectable HI titer.

Discussion:

Avian influenza H5N1 virus remains in the focus of global concern due to its nature of high degree of genetic shift and drift. Controlling this pandemic disease needs beside the standard biosecurity measures, an effective vaccine protocol. Among which is the inactivated autologus vaccines prepared from the local field isolates which needs rapid, cost effective and speedy production scales. This study described the propagation of H5N1 Egyptian field isolate designed as (A/chicken/Gharbia/ch100/2010(H5N1)) on CEF cells. First virus isolated from an outbreak in Gharbia governorate during early 2010. The

H5- specific RT-PCR amplified an amplicon with the expected size (315 bp) indicating the nature of the virus (Yuen *et al.,* 1998). Field Isolated virus , SPF-egg and tissue culture propagated viruses was characterized by the amplification of the315 bp fragment of the cleavage site of H5 gene , as noticed in fig1 a clear band was amplified using the specific primer set that amplify the cleavage site of the *h5* gene. The virus was tested for purity using HI test utilizing Monospecific antisera against both Newcastle disease virus and egg drop syndrome virus (the most Hemaglutinating viruses) and no reaction was found .

The amplicon was further characterized by nucleotide sequencing and alignment with the published sequence of the 2009 isolate. As shown in fig 2, there is great homology between the 2 sequences (the primer annealing sequence was omitted from the alignment).

In order to establish a protocol for tissue culture propagation of AI, the egg propagated isolated strain was used to inoculate CEF. The time coarse of Tissue culture propagation of the virus was estimated. After 2 days of inoculation, the CPE begin to appear and the complete destruction of the monolayer was seen after 5 days post inoculation. serial passage of the virus (3^{rd} passage) gave a comparable high titer (8 Log2) which is a suitable titer for production of inactivated vaccine. Other studies showed that propagation of AI on mammalian tissue culture like MCDK could yield more virus titer due to the fact that HA cleavability is defined by the sensitivity of the HA to proteases, suggesting that the tissue distribution of specific proteases could be a co-determinant of virulence. Differences in the plaque-forming ability in CEF, MDBK, and MDCK cell cultures may reflect variations in the intracellular proteases. suggested that mammalian cells, such as MDCK cells, may contain proteases with a broader substrate specificity than those found in avian cells CEF (Kira *et al.,* 2010).

The first reported isolation of the Gs/Gd-like lineage viruses, which have multiple basic amino acids at the HA cleavage site and are highly pathogenic for chickens, was obtained from a goose in China in 1996 (Xu *et al.*, 1999). Since then, H5N1 AI viruses from Asia have shown considerable variation in their internal genes through reassortment with other AI viruses and continuous evolution of the HA and NA genes. A phylogenetic analysis showed that (A/chicken/Gharbia/ch100/2010(H5N1)) and 2009 field isolates are differ in the amino acid at position 225 (table 1) and the first 3 amino acids of Egyptian isolates were completely differe from the other strains isolated in the early epidemics in Asia.

Like many other enveloped viruses, they code for a surface glycoprotein that must be cleaved by cellular proteases for activation. HA, a major influenza surface glycoprotein, is translated as a single protein, HA_0. For viral activation, HA_0 (assembled as trimers) must be cleaved by a trypsin-like serine endoprotease at a specific site, normally coded for by a single basic amino acid (usually arginine) between the HA1 and HA2 domains of the protein. After cleavage, the two disulfide-bonded protein domains produce the mature form of the protein subunits as a prerequisite for the conformational change necessary for fusion and hence viral infectivity (Lamb and Krug, 1996).

In aquatic birds, normal influenza replication takes place in the intestinal tract and tends not to cause symptoms. In mammals like humans and swine, influenza replication is limited to epithelial cells of the upper and lower respiratory tract. This tissue tropism is controlled to some extent by the limited expression of the appropriate protease for viral activation by HA cleavage. In mammals, the suspected protease in the respiratory tract is tryptase Clara, a serine protease produced by nonciliated Clara cells of the bronchial and bronchiolar epithelia (Rott *et al.*, 1995). Occasional avian influenza strains with an insertion mutation at the cledvavage site of HA, allowing HA to be cleaved by ubiquitously expressed proteases (furin and other subtilisin family proteases), as a consequence, the virus can replicate throughout the bird's body, producing necrotic foci in spleen, liver, lung, and kidney and encephalitic lesions in brain (Easterday and tumova 1978 and Kin *et al.*, 2009).

These highly virulent strains have been observed in only two of the 14 described HA subtypes in birds that emerge only occasionally but can cause devastating mortality in poultry flocks (Horimoto *et al.*, 1995). The insertion responsible for the ubiquitous cleavage adds additional basic amino acids at the cleavage site (Senne *et al.*, 1996), with a minimal motif of R/L-X-R/L-R. Until recently, this mutation had been found only in avian viruses of the H5 and H7 subtypes, subtypes that were not thought to infect humans. This barrier was broken dramatically in 1997 in Hong Kong when 16 people were infected with an avian H5N1 influenza virus (CDC reports 1998)

One of the properties of AI strains is its inability to undergo HA cleavage activation in tissue culture without the addition of exogenous trypsin although some studies showed that some strains (A/WSN/33/H1N1 which responsible for the 1918 epidemic) could undergoes HA cleaved by serum plasmin (Lazarowitz, *et al.* 1973)

Accounting to these knowledge, the virus propagates on Tissue culture will not be subjected to the enzymatic activation pathway resulting in loss of the HA properties. In this study, the propagated virus on tissue culture was subjected to different concentration and incubation time of trypsin and then the virus was tested for the HA properties . It was found that incubation of the virus with 0.5µg/ml trypsin for 10 min at 37°C resulted in complete regain of HA activity of the virus as seen in table (2).

In conclusion, the tissue culture propagation of the field isolate of HPAIV could be a cost effective and more suitable method for production a high titer virus particles. The HA activity of the virus was found to be easily regained after TC propagation by pretreatment of the virus with trypsin.

Acknowledgement:

This research paper is supporetd by the project titled "*The protective efficacy and Immunogenicity of DNA vaccine coding for H5 gene of avian influenza virus (H5N1)*" Funded by the STDF.

The author also would acknowledge Dr. Lamiaa Omar head of Q.C. of inactivated viral poultry vaccine CLEVB for providing the standard monospecific antisera used in this study. I am also grateful to Dr. Ahmed Maher Biotechnology Department CLEVB for providing the tracheal samples used for isolation of the virus.

REFERENCES

Arafa, A.; Suarez, D. L.; Hassan, M. K. and Aly, M. M. (2010): Phylogenetic analysis of hemagglutinin and neuraminidase genes of highly pathogenic avian influenza H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogeneity with multiple distinct sublineages. Avian Dis. Mar; 54(1 Suppl):345-349.

Bahgat, M.M.; Kutkat, M.A.; Nasraa, M.H.; Mostafa, A.; Webby, R,; Bahgat, I.M and Ali, M.A.A. (2009): Characterization of an avian influenza virus H5N1 Egyptian isolate J. Virol. Methods, 159: 244 –250

Catherine I. T.; Barclay, W. S.; Zambon, M. C. and Pickles, R.J. (2006): Infection of Human Airway Epithelium by Human and Avian Strains of Influenza A Virus J. OF VIROL, (80), 16: 8060–8068

Centers for Disease Control (1998) Update: Isolation of avian influenza A (H5N1) viruses from human – Hong Kong, 1997 – 1998 Morb. Mortal. Wkly Rep. 46: 1245–1247.

Easterday, B. C. & Tumova, B. (1978) : in Diseases of Poultry, ed. Hofstad, M. S. (Iowa Stae Univ. Press, Ames, IA), 7th Ed., pp. 563–565.

Fouchier, R. A. Munster, .V.; Wallensten,A.; Bestebroer,T.M.; Herfst,S,; Smith,D.; Rimmelzwaan,G.F.; Olsen,B. and. Osterhaus,A.D. (2005): Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol., 79: 2814–2822.

GOVS. 2009. Plan for controlling Avian Influenza. General Organisation of Veterinary Services and Ministry of Agriculture and Land Reclamation. Arab Republic of Egypt.

Hoffman, E.; Krauss, S.; Perez, D.; Webby, R. and Webster,R.G. 2002: Eight-plasmid system for rapid generation of influenza vaccine vaccine, 20:3156-3170

Horimoto, T.; Rivera, E.; Pearson, J.; Senne, D.; Krauss, S.; Kawaoka, Y. and Webster, R. G. (1995) Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 213, 223–230.

Katz, J, Hancock, K, Veguilla, V, Zhong, W, Lu, XH, Sun, H, Butler, E, Dong, L, , Liu, F, Li, ZN, , DeVos, J, Gargiullo, P, Cox, N (2009). Serum Cross-Reactive Antibody Response to a Novel Influenza A (H1N1) Virus After Vaccination with Seasonal Influenza Vaccine Morbid. Mortal. Weekly Rep., 58 (19), 521-524

Killian, M.L. (2008): Hemagglutination Assay for the Avian Influenza Virus Methods in Molecular Biology (436): 47-52

Kin, M. D.; Guentzel, M. N.; Arulanandam, B. P. ; Lupiani, B. and J. P. Chambers (2009.) Proteolytic bacteria in the lower digestive tract of poultry may affect avian influenza virus pathogenicity Poult Sci. 88:1388-1393

Kira, A. M.; David, E. ; Stallknecht,B. and Swayne,D.E (2010) Evaluation and Attempted Optimization of Avian Embryos and Cell Culture Methods for Efficient Isolation and Propagation of Low Pathogenicity Avian Influenza Viruses Avian Diseases 54:622–626

Lamb, R. A. and Krug, R. M. (1996) : in Fields Virology, eds. Fields, B. N, Knipe, D. M & Howley, P. M. (Lippincott- Raven, Philadelphia), 3rd Ed., pp. 1359–1362.

Lazarowitz, S. G., Goldberg, A. R. & Choppin, P. W. (1973) Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: Host cell activation of serum plasminogen Virology 56, 172–180.

Lee, C.; David, L. S.; Terrence, M. T.; Sung, H.W.; Kwon ,Y.K.; Lee ,Y. J.; Choi ,J.G.; Joh, J.S.; Kim, M.C.; Lee, E.K.; Park , J.M.; Lu , X.; Jacqueline , M. K.; Spackman , E.; Swayne , D.E. and Kim, J.H.(2005): Characterization of Highly Pathogenic H5N1 Avian Influenza A Viruses Isolated from South Korea J. virol 3 : 3692–3702

OIE 2008. update on highly pathogenic avian influenza.in animals july 1st 2008

Potter, CW, and Oxford, JS (1979). Determinants of immunity to influenza infection in man. Br Med Bull, 35: 69-75

Reed, L.J. and Muench, H. (1938): A simple method for estimating fifty percent endpoints. American Journal of Hygiene, 27: 493-500

Rott, R., Klenk, H.-D., Nagai, Y. & Tashiro, M. (1995) Influenza virus cell enzymes and pathogenicity. Am. J. Respir. Crit. Care Med. 152: 516–519.

Senne, D. A., Panigrahy, B., Kawaoka, Y., Pearson, J. E., Su¨ss, J., Lipkind, M., Kida, H. & Webster, R. G. (1996) Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis. 40, 425–437.

Swayne, D. E.; Perdus, M. L.; Garcia, M.; Rivera-Cruz, R. and Brug, M., 1997 Pathogenicity and diagnosis of H5N2 Mexican avian influenza viruses in chickens. Avian Dis. , 41: 335–346.

Xu, X. ; Subbarao, K.; Cox, J, and Guo. Y. (1999): Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: Similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 261:15-19

Yuen, K.Y.; Chan, P.K.; Peiris, M.; Tsang, D.N.; Que, T.L.; Shortidge, K.F.; Cheung, P.T.; To, W.K. ; Ho, E.T.F. ; Sung, R. and Cheng,

A.F.B.(1998): Clinical feature and rapid viral diagnosis of human diseases associated with avian influenza A H5N1 virus. Lancet 351: 467-471.

تحور و تمرير فيروس إنفلونزا الطيور و شديد الضراوة من النوع H5N1 فى خلال الزرع النسيجى

يوسف عادل سليمان، عفاف خضير، نرمين محمود السعيد، منصور هاشم عبد الباقى، إلهام عطا الإبيارى

المعمل المركزى للرقابة على المستحضرات البيولوجية البيطرية

فيروس انفلونزا الطيور عادة ما يشير إلى الأنفلونزا من النوع A من الفيروسات الموجودة أساسا في الطيور، ولكن يمكن أن تحدث العدوى بين البشر. لمعظم الناس عموما المخاطر منخفضة من الإصابة بالفيروس ، لأن الفيروسات لا تصيب البشر عادة. ومع ذلك، هناك حالات من العدوى البشرية بالفيروس H5N1 تم الإبلاغ عنها منذ ١٩٩٧. وفي الدراسة الحالية، تم عزل سلالة شديدة الضراوة من أنفلونزا الطيور H5N1، وأكدته باختبار البي سي ار و تحليل التتابع الجيني لموقع الانشطار. وكشف التحليل درجة عالية من الهوية الجينية مع التتابع الجيني فيروس H5N1 عزل من مصر في وقت سابق. وكان التشابه في الأحماض الأمينية للفيروس معزولة مماثلة لسلالات الفيروس H5N1 الشديد الإمراض (G-E/R-R-R/K-K-R) وغيره من الفيروسات المعزولة في مصر في السنوات السابقة وخاصة في وجود الأحماض الأمينية أرجينين في موقع الانشطار للبروتين هيماغلوتينين.

تتابع موقع الانشطار للعترة المعزولة من الفيروس H5N1 تم وضعه في بنك الجينات تحت مسمي A/chicken/1000CLEVB/2010

تم كذلك زراعة الفيروس علي الخلايا الليفية لأجنة الدجاج الخالي من المسببات المرضية. وكان عيار الفيروس يزداد بسرعة من 8.5 $\log_{10}$ /ml في المرة الأولي من التمرير إلى 4.8 $\log_{10}$ /ml في المرة الثالثة.

و تم استعادة خاصية تلزن الدم بمعالجة الفيروس المستخلص من زراعة الأنسجة عن طريق العلاج التربسين 0.5µg/ 10 min

و هذه الدراسة تفتح أبواب لإنتاج لقاح مثبط نامي علي خلايا الزرع النسيجي .