Genetic Characterization of Avian Influenza Virus Isolates of Sharqiyah Province - Egypt, 2011.

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Abstract: Two hundred and twenty cloacal, tracheal swabs and tissue samples from different poultry species (chicken and duck) either in commercial breeding or in backyard system showed respiratory manifestations with variable mortalities were collected from different localities in Sharqiyah province during 2011. The tested samples were inoculated into the allantoic cavities of 9-11 days old specific pathogen free embryonated chicken eggs (SPF-ECE) for virus isolation. The harvested allantoic fluids were tested for detection of hemagglutinating viruses using slide and plate haemagglutination test followed by subtyping using haemagglutination inhibition test and genotyping using RT-PCR. All AIV isolates were proved to be H5N1avian influenza virus. Partial hemagglutinin (HA) gene sequencing was also done, and the sequences of these isolates were compared with some available Egyptian published sequences in Genbank and the sequences of currently used imported vaccinal strains in Egypt as Volvac Avian Influenza Killed Virus (AI KV) H5N2 (A/Chicken/Mexico/232/94) vaccine with accession number (AY497096.1) and YEBIO H5N1 (A/Goose/Guangdong/96) vaccine (Re-1) with accession number (AF144305.1). Sequencing results revealed 96-100% homology of H5 gene with previously published sequences of H5N1 isolates of Egypt from 2006 -2011, in addition, the percent of identity between our isolate and Chinese vaccinal strain was 40.6% and 41% with the Mexican vaccine. While the percent of identity of some reference Egyptian isolates in 2006 with the Mexican vaccine was 78.2% and 92.9% with the Chinese vaccine. Phylogenetic analysis showed independent sub-clustering of the two viruses (A/ck/Faquos/amn12/011 and A/dk/Zagazig/amn13/011 within the Egyptian sequences that may indicate a possible differential adaptation in the two hosts. The positive AIV (H5N1) isolates were passaged on different cell lines of avian and mammalian origin to determine the differential susceptibility of present isolates on these cell lines. The results showed that the isolates can produce a substantial cytopathic effect within 3 days of infection after addition of trypsin (2µg/ml) on CEF while after 3 passages of adaptation on both Vero and MDBK cells. Our study results showed that the currently used imported vaccinal strains in Egypt cannot give a good protection level due to high mutation rate that necessate for production of autogenous vaccine from freshly local isolates. In addition CEF, Vero and MDBK cells can be used as alternative systems for AIV isolation avoiding viral mutation occurred in SPF-ECE but further studies are needed to determine the best cell line that cannot produce any mutational changes during isolation step to help the authorities for production of cell culture adapted inactivated vaccine from freshly local isolates to control the current outbreaks.

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1. Introduction

Avian influenza (AI) is a viral disease spread worldwide caused by influenza A viruses of the Family Orthomyxoviridae. Influenza viruses are classified into16 subtypes on the basis of the surface glycoprotein hemagglutinin (HA) and 9 subtypes on the basis of the glycoprotein neuraminidase (NA) **(Fouchier *et al.,* 2005)**. On the basis of the severity of clinical signs and mortality rate in experimentally inoculated chickens, avian influenza virus (AIV) can be categorized into 2 pathotypes: high-pathogenicity avian influenza (HPAI) and low-pathogenicity avian influenza (LPAI) **(Cattoli *et al.,* 2004)**. In Egypt, poultry possesses considerable importance as a source of animal protein for human consumption. Before the incursion of HPAI (H5N1) to Egypt in 2006, a huge poultry population of about 1 billion heads was reared for home consumption and trade to other Middle East and African countries **(Aly *et al.,* 2008)**. About 75% of the poultry was raised in commercial farms. Keeping of mixed backyard poultry flocks were amounted to about 25% of the countries' poultry population and is intimately intertwined with human rural and urban life **(Saad *et al.,* 2007)**. In mid-February, 2006 a devastating HPAI H5N1 infected the commercial poultry production sectors and backyards in Egypt **(Aly *et al.,* 2006 a, b)**. In Africa, H5N1 HPAI infection of domestic birds was reported first in Nigeria in early 2006 and subsequently in Egypt, Niger, Cameroon, Burkina Faso, Sudan, Cote d'Ivoire, and Togo **(WHO, 2010a)**. Egypt has been most severely affected by continuous outbreaks, resulting in severe losses in the poultry industry **(WHO, 2010 a&b)**. As of July 2008, Egypt reported outbreaks in nine Governorates (Gharbiyah, Minufiyah, Kafr El- Shaykh, Daqahliyah, Sharqiyah, Minya, Jizah, Suhaj, and Luxor) in commercial and backyard poultry and,

poultry in live bird markets from 7 February to 14 June 2008 **(WHO, 2010 a &b)**. Following the widespread of HPAI (H5N1) in Egypt, authorities began culling and vaccination to control the spread of the disease in poultry. However, even three doses of vaccine (inactivated oil–whole virus emulsion H5N1 vaccines imported in a trial to control the H5N1 in Egypt from China and Europe as Vaccines Mexico/H5N2, and the Chinese reassorted vaccine Re-5/H5N1 have failed to provide the expected level of protection against the currently circulating clade 2.2.1 H5N1 viruses due to antigenically distantly related vaccine strains **(Peyre *et al.*, 2009)**. Avian influenza vaccine induced protection was both dose- and vaccine strain-dependent **(Swayne *et al.*, 2001)**. In the present study, we characterize H5N1 isolates from different localities of poultry populations in Sharqiyah Province using the tracheal, cloacal swabs and tissue samples of naturally infected chickens and ducks collected during 2011 to get a deep insight into possible intra- and inter-subtype sequence variations. The genetic and phylogenetic properties of isolated viruses were determined and compared to some available Egyptian published sequences and imported vaccinal strains commony used in vaccination of our flocks to determine the vigor of the mutational events of HA hoping that it will help the authorities for production of autogenous vaccine from freshly local isolates to control the current outbreaks.

2. Materials and Methods

Sampling

The study was carried out in different localities within Sharqyiah Province, Egypt during 2011. Freshly dead and sick birds from infected chickens and ducks were collected, 140 tissue samples (120 from broiler farm, 10 from backyard ducks and 10 from backyard chickens), 30 cloacal swabs from broiler chickens, 5 from backyard ducks and 10 from backyard chickens. In addition to, 30 tracheal swabs from broiler farm, 5 from Backyard ducks and 5 from backyard chickens). These samples were taken on virus transport medium (VTM) composed of sterile Phosphate Buffered Saline (PBS), in addition to antibiotic mixture, contain 100IU/ml Penicillin, 100µg/ml Streptomycin (Pen-Strept, Gibco, Invitrogen, Code 4512), 250µg/ml Gentamycin and 10% glycerol. Samples were stored at 80% until processed according to **Lee and Suarez, 2004)**.

Virus isolation in specific pathogen free embryonated chicken eggs (SPF-ECE)

SPF-ECEs were purchased from poultry farm at Qom Osheem- Al Fayoum, Egypt. A volume of 100ul from each prepared samples was inoculated into three 9 days-old (SPF-ECE) via the allantoic cavity according to (**Payungporn *et al.*, 2006)**. The harvested allantoic fluid (AF) was tested for the presence of haemagglutinating activity according to **(Beard, 1989)**. HA-positive allantoic fluids were subtyped by haemagglutinin inhibition (HI) test.

Subtyping of AIV isolates by hemagglutination inhibition (HI) test

The test was conducted according to **OIE(2008)** using reference H5N1 antiserum and reference H5N1 AIV that were kindly supplied by Veterinary Serum and Research Institute, Abbassia, Cairo (VSVRI). Positive HI samples were moleculary confirmed by RT-PCR using H5 primers, while negative results were retested by RT-PCR using primers against H7 &H9.

Genotyping of AIV isolates by RT-PCR

RT-PCR was used for identification of the AI viruses depending on three sets of primers that specifically amplify the H gene of H7, H9, and H5 subtypes of AIV which listed in (**Table 1)** were previously designed by **(Rashid *et al.*, 2009)** and synthesized by Integrated DNA Biotechnologies (IDT). The primers were aliquoted to a final concentration of 100 *p*mol /µL and stored at −20°C until further use. The technique was started with the RNA extraction from the collected allantoic fluids using RNA extraction kit (Gene JET^{TM} RNA Purification Kit, # K-0731 (Fermentas)) according to instructions of the manufacturer. Extracted RNAs were amplified using a One-Step reverse transcription-PCR (RT-PCR) kit (Gene JET^{TM} RNA Purification Kit, # K- 0731 (Fermentas)) to amplify HA genomic segments as partial length. The reaction was carried out in an Eppendorff thermal cycler (MWG, BIOTECH). (Program profile: cycle: 15 minutes at 50 ^{o}C (RT-reaction); cycle at 95 ^{o}C for 2 minutes (initial denaturation); 40cycles (of denaturation at 95 ^{o}C for 20 seconds, of annealing 50 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1 minute) and cycle of final extension at 72 ^{o}C for 5 minutes). The DNA amplicons were visualized using 2% agarose gels with ethidium bromide with standard 1-kb DNA Marker (Fermentas) at 120 V for 20 min. Amplified products were visualised by ultraviolet light transillumination (Spectroline, model : TVC-312R/F). Negative controls were included in each assay for detection of any contamination.

Sequence analysis

PCR products were purified with (Gene JET PCR purification kit, #K0701, Fermentas). Each purified amplicon was sequenced in both forward and reverse directions. The sequencing reaction was performed by (Macrogen Inc., Korea AB13730XL machine).

Table (1): Oligonucleotide primers for subtyping AIV haemagglutinin using RT- PCR

Name of oligonucleotide	Sequence primer (5'- 3')	Product length (bp)
H5-F H5-R	5'-ACT ATG AAG AAT TGA AAC ACC T-3' 5'-GCA ATG AAA TTT CCA TTA CTC TC3'	456
H7-F H7-R	5'-ACA TAC AGT GGG ATA AGA ACC-3' 5-TCT CCT TGT GCA TTT TGA TGC C-3'	300
H9-F H9-R	5'-AGC AAA AGC AGG GGA AYW WC-3' 5'-CCA TAC CAT GGG GCA ATT AG-3'	808

Codes for mixed bases position: Y, C/T; R, A/G; W, A/T; B, G/C/T; K, G/T.

Multiple sequence analysis and phylogenetic tree

BLAST analyses were initially performed to establish HA sequence identities to GenBank accessions **(Altschul *et al.,* 1990)**. Comparative analyses and phylogenetic trees were performed using the Lasergene.7 (software www.dnastar.com/t-products-lasergene.aspx) between sequences derived from this study and some sequences posted in Genbank for other Egyptian sequences from 2006-2011 (**Table 2)** and sequence of currently used imported vaccinal strains in Egypt as Volvac Avian Influenza Killed Virus (AI KV) H5N2 (A/Chicken/Mexico/232/94) vaccine with accession number (AY497096.1) and YEBIO H5N1 (A/Goose/Guangdong/96) vaccine (Re-1) with accession number (AF144305). The liability of internal branches was assessed by 1000 bootstrap replications and p-distance substitution model **(Siddique *et al.,* 2012)**. The nucleotide sequences presented in this article have been deposited in the GenBank database under accession numbers (Accession No: JQ627585 & JQ627586).

Table (2): Accession numbers of previously published sequences of H5N1 isolates of Egypt from 2006 -2011 used in this study.

Category	2011	2010	2009	2008	2007	2006
Accession numbers	JN807867.1	JN807801.1	CY062457.1	GQ184241.1	EU095025.1	EU146866.1
	JN807865.1	JN807797.1	CY062449.1	GQ184242.1	EF535826.1	EF469651.1
	JN807863.1	JN807788.1	HQ198254.1	FJ226057.1	EU183329.1	EF441276.1
	JN807853.1	CY099578.2	GU002699.1	GU811716.1	EU183331.1	EU146868.1
	JN807857.1	CY099590.1	GU002697.1	GU811715.1	EF469657.1	EF469656.1
	JN807859.1	CY099589.1	GU002691.1	GU811711.1	EU373737.1	CY016906.1

Susceptibility of positive AIV H5 isolates on cell cultures /cell lines

The cell cultures that were selected for this study were African green monkey cells (Vero), Madin Darby-Bovine Kidney cells (MDBK) and chick embryo fibroblast cells (CEF). Both Vero and MDBK cells were kindly supplied by (VSVRI, Abassia, Cairo, Egypt) and chicken embryo fibroblast (CEF) primary cultures were prepared from 3-5 SPF-ECE according to (Tomo *et al.,* 2008). Cell cultures propagated in Dulbecco's modified eagle media (DMEM) supplemented with 5% fetal bovine serum (FBS) and antibiotic/antimycotic solution at a concentration of 1X. All cell cultures were grown and maintained at 37 ^{o}C in presence of 5% CO_2. The cells were seeded into two 24-well cell culture plates and allowed to form confluent monolayer overnight.Virus inoculation occured when the monolayers showed 90% - 95% confluency. Each diluted virus sample (10^{-2} is dilution used for inoculation of each viral sample at dose of 0.2ml /well) was inoculated onto two wells of 24-well cell culture plates of each cell culture with addition of trypsin (2µg/ml viral suspension). The cells were placed into the incubator at 37 ^{o}C for 3 days with microscopic examination twice daily for the detection of cytopathic effect (CPE) according to (Kira *et al.,* 2010).

3. Results

Isolation of AIV in SPF -ECE:

The inoculated chicken embryos died within 24-48 hours post inoculation with hemorrhagic embryos. All harvested allantoic fluids were submitted to slide and plate HA to test presence of hemagglutinating viruses in the samples. Haemagglutinating viruses were isolated from 65 tissue samples (61 samples from broiler farms, 2 from Backyard chickens and 2 from Backyard ducks) out of 140 collected tissue samples. Also, 7 cloacal samples (3 samples from Backyard ducks, 2 from Backyard chickens and 2 from broiler farms) out of 40 cloacal swabs and 12 tracheal swabs (10 from broiler farms and 2 from backyard chickens) out of 40 collected tracheal swabs from clinically infected birds.

Subtyping of AIV isolates using HI test:

Subtype analysis of virus isolates from these samples using HI test, showed that most of isolates belonged to the H5N1 subtype of AIV. In broiler

chickens, AIV subtype H5 was detected in 40 tissue samples out of (61 positive HA samples), 5 tracheal samples out of (10 positive HA samples) and 1 cloacal swab out of (2 positive HA samples). In backyard samples, AIV subtype H5 was detected in 2 tissue samples of Backyard chickens out of (2 positive HA samples) and in 1 tissue samples of Backyard ducks out of (2 positive HA samples), 1 tracheal swab from Backyard chickens out of (2 positive HA samples) and 2 cloacal swabs from Backyard ducks out of (3 positive HA samples) with no detection in that of Backyard chickens.

Genotyping of AIV isolates by RT-PCR:

Most of isolates were H5-AIV positive using HI test. Also, these results were confirmed by RT-PCR using H5-primers, where they yielded a sharp band of the expected size 456 bp (Figure 1). Negative HI isolates were tested again by RT-PCR using primers for H7& H9 AIV genes. However, no detection for H7or H9 was observed. Out of 15 pooled samples, 11 samples were found to be positive for AIV subtype H5 with no detection of H7 and or H9.

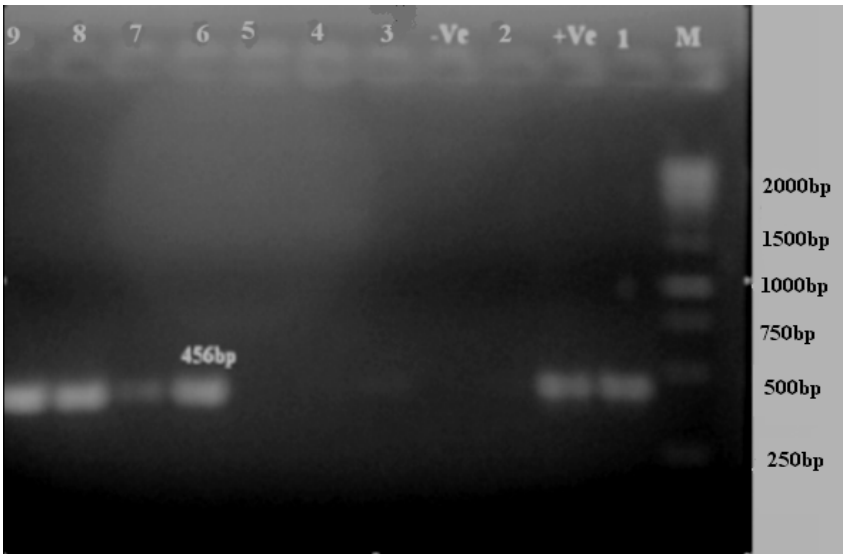

Figure (1): RT-PCR analysis of AIV subtype H5 using specific primers for partial sequences of the H5gene. The amplification products for H5 appeared at the expected molecular weights of 456 bp. M: Molecular size Marker (1 kb), Lane (+Ve) is positive control AIV subtype H5. Lanes 1,6,7,8, 9 are positive clinical samples. Lanes 2, 3, 4, and 5 are negative clinical samples and Lane (-Ve) is negative control (PCR reagents without template)

Genetic analysis of the isolated H5 AIV

Phylogenetic tree pattern of the alignment for nucleotide sequence of AIV isolate of present study (A/chicken/Faquos/amn12/2011(H5N1) and (A/Duck/Zagazig/amn13/2011 (H5N1) with accession no. (JQ627585 & JQ627586), respectively were compared with some available Egyptian published sequences in GenBank and the sequence of two currently used imported vaccinal strains in Egypt. The alignment was constructed using MegAlign program (Lasergene.v.7 software, www.dnastar.com/t-products-lasergene.aspx). The results showed that there are low percent of identity between isolate in the present study and two currently used imported vaccinal strains in Egypt.The identity percent was 40.6% with the Mexican vaccine and 41% with the Chinese one while the percent of identity of some reference Egyptian isolates isolated in 2006 with the Mexican vaccine was 78.2% and 92.9% with the Chinese vaccine (Data not shown). In addition, phylogenetic analysis showed independent sub-clustering of the two viruses (A/chicken/Faquos/amn12/2011(H5N1) and A/duck/Zagazig/amn13/2011 (H5N1) within the Egyptian sequences that may indicate a possible differential adaptation in the two hosts **(Figure 2)**.

Susceptibility of positive AIV H5 isolates on cell cultures /cell line

The CPE induced by positive AIV H5 isolates were in the form of cell rounding on Vero cells appeared 36 hours post inoculation after 3 passages with continous addition of trypsin **(Fig. 3 A & B).** While positive AIV H5 isolates induced detached cell sheet on MDBK cells 36 hours post inoculation after 3 passages **(Fig. 4 A & B)**. Morover, The CPE induced on CEF was inform of focal foci with cell damage 36 hours post inoculation (**Fig. 5 A & B)**.

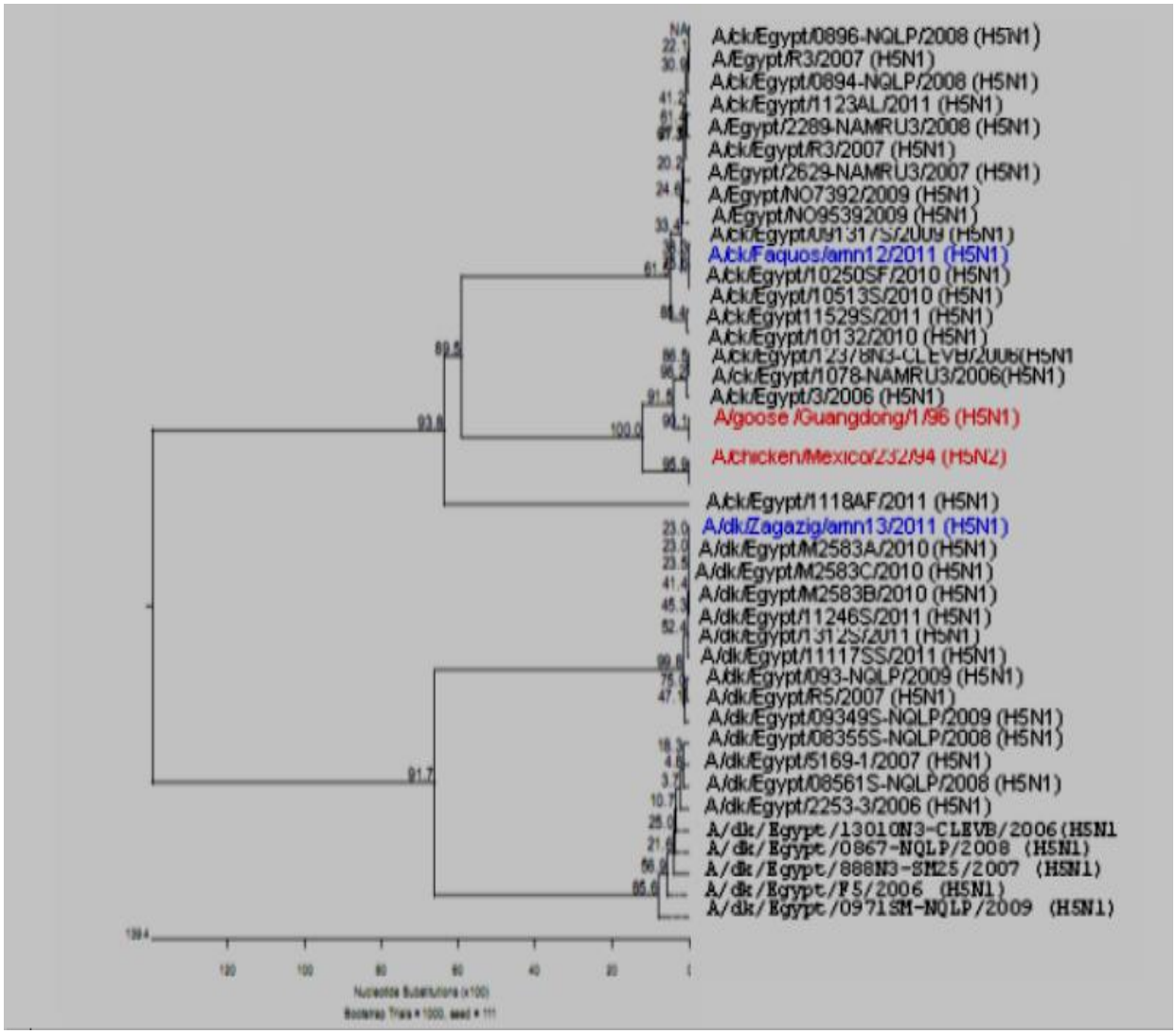

Fig. (2): Phylogenetic tree of virus isolates (A/chicken/Faquos/amn12/2011(H5N1), (A/duck/Zagazig/amn13/2011 (H5N1) and currently used vaccines with some available Egyptian published sequences that were taken from the Genbank database. The neighbor-joning trees based on partial length nucleotide were generated with MegAlign program with 1000 bootstrap value.

*The virus isolates sequence are indicated in blue color and two vaccinal strains are indicated in red color.

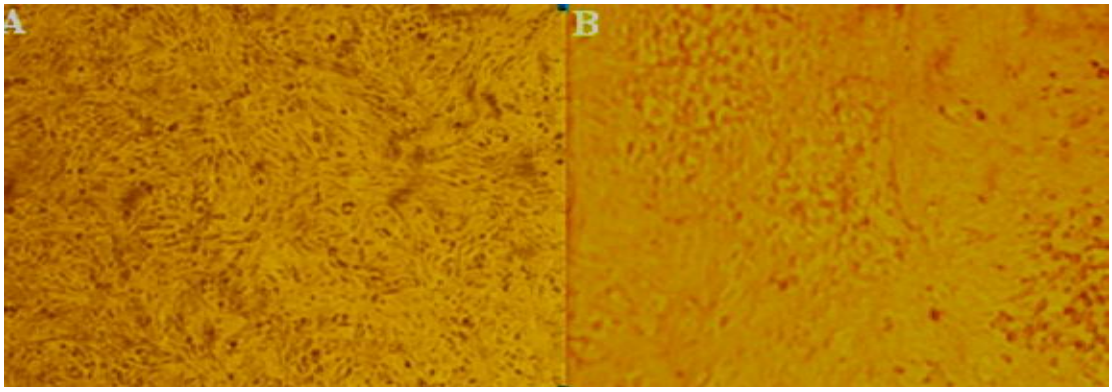

Figure (3): A; Normal Vero cell line showing confluent monolayer cell sheet (X 100). B; Vero monolayer cell sheet inoculated with field isolate, after 36 hours it showed typical rounding of cells (X 100).

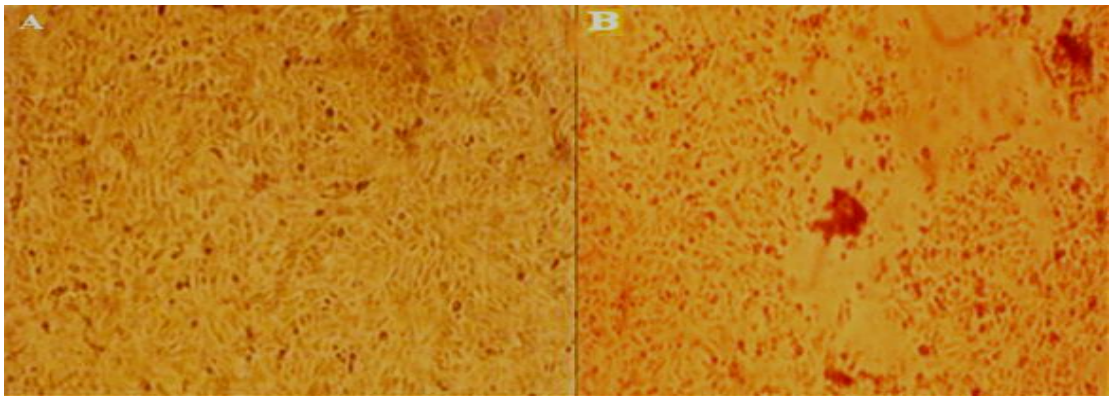

Figure (4): A; Normal (MDBK) cell line showing confluent monolayer cell sheet (X 100). B; MDBK monolayer cell sheet inoculated with field isolate, after 36 hours it showed detachment of cells from cell sheet (X 100).

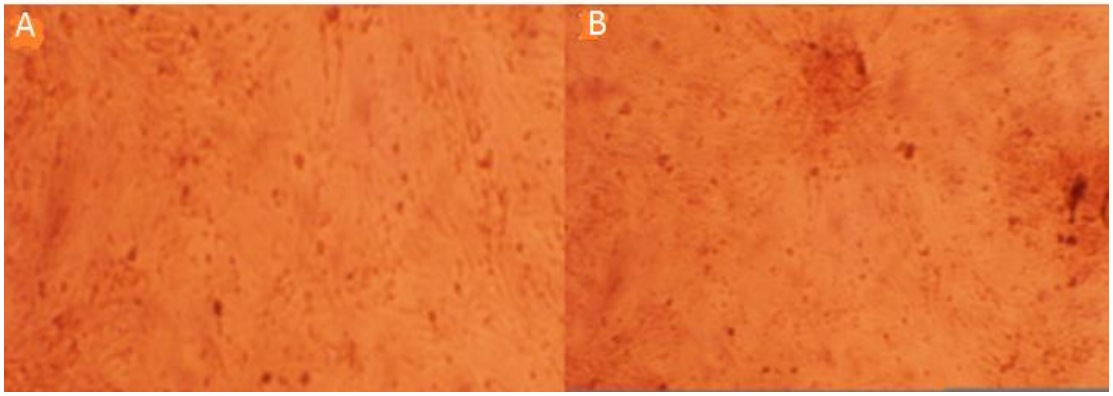

Figure (5): A; Normal CEF cell showing confluent monolayer cell sheet (X 100). B; CEF monolayer cells inoculated with field isolate, after 36 hours it showed focal foci CPE and cell damage (X 100).

4. Discussion

Avian influenza virus (**AIV**) infection is a devastating viral disease causing enormous losses in the poultry industry worldwide (**Capua and Alexander, 2004**). Rapid and definite diagnosis of H5N1 virus plays a central role in depopulation of infected flocks and controls the spread of the infection to contact birds and humans (**Spackman** *et al.,* **2002**). In the current study, AIV H5N1 strain was isolated from infected poultry broiler farms and backyards suffering from respiratory distress and variable mortalities in different localities within Sharqyiah Province during 2011. The infected allantoic fluid showed positive haemagglutination titers when tested with chicken RBCs and subtype identification of the viruses was determined by standard HI test. RT-PCR and Sequencing of the partial length haemagglutingin were performed and compared with some available Egyptian published sequences in the flu database and two currently used imported vaccinal strains in Egypt as (A/chicken/Mexico/232/94(H5N2)) and (A/Goose/Guangdong/1/96(H5N1)). The AIV was isolated from cloacal, tracheal swabs and tissue samples that were inoculated in 9day-old SPF-ECE. All embryos died within 24-48 hrs post inoculation. The virus was isolated from 84 out of 220 samples (38.1%). The number of virus passages in eggs during isolation was limited to one in order to restrict genome modifications linked to laboratory manipulation according to (Schild, 1983). Virus isolation techniques is considered the reference standard for the diagnosis of AIV in the collected samples during a period of 2006-2010 in Egypt (Dalia *et al.,* 2011). This was agreed with (Swayne *et al.,* 1998) who told that virus isolation (VI) is the best test method to accurately identify the presence of AIV from tracheal and cloacal swab samples. VI is necessary to confirm the presence of the virus in an index case and to perform characterization of the virus. (Woolcock *et al.,* 2001). The positive samples for HA was then tested by HI test to differentiate AIV from other haemagglurinating viruses (Beard, 1989). Screening of 84 allantoic fluids samples (were positive in HA) revealed 52 positive samples using HI test (61.9%), this agreed with (Webster *et al.,* 2002) who said that VI is very sensitive, but not highly specific or selective because other viruses that may be present in poultry samples can grow in chicken embryos. Historically, virus isolation in embryonated chicken eggs (ECEs) is the standard technique of virus detection and propagation

of viruses followed by antigenic characterization of the circulating strains using classical methods of hemagglutination inhibition (HI) (Pearson, 2003). Because these standard methods are costly and requires much forethought concerning scheduling because embryos must be incubated 9–11 days prior to use (Spackmann *et al.,* 2008). In addition, the persistent propagation of AIV in ECEs has been shown to lead to the emergence of mutations in the hemagglutinin glycoprotein (Schild, 1983). The RT-PCR is rapid, it detects various influenza A strains from different species and is at least as sensitive as traditional methods of virus isolation using ECE followed by serological identification(Munch *et al.,* 2001). However, due to frequent mutations in influenza viruses, the sequence of primer sets used in PCR-based detection must be appropriate for the detection of currently circulating strains (Fouchier *et al.,* 2005). All positive HI tested samples were identified molecularly by RT-PCR using primers for H5, H7and H9 gene. All positive samples were AIV subtype H5 only(of 456 bp size fragments) with no detection for H7 and or H9 subtypes (Fig. 1) and these results agreed with that obtained by Aly *et al.* (2008) were in August (2007), the Egyptian Government reported the isolation of AI subtype H7 from wild migratory ducks in El-Abassa lake, El- Sharkia Province in addition to the results of serological and molecular surveillances on H7 backyard from eleven villages in close contact to the lake were negative providing an evidence of the absence of H7avian influenza in domesticated birds. Comparative analysis was performed using Lasergene.7 software (www.dnastar.com/t-products-lasergene.aspx). The results indicated that the Egyptian viruses continuously evolving in different clusters from 2006 till 2011 as shown in (Fig. 2). This was agreed with Dalia *et al.* (2011) who recorded that new outbreaks of H5N1 occurred in the different years from 2006- 2010, indicating that the virus is circulating in the region. So the emergence of these variant strains and their spread in a short period of time to several Governorates in Egypt is considerable antigenic variation from pervious Egyptian isolates emphasizes the need for continuous monitoring of genetics and antigenic changes in HPAI H5N1 as early as warning system of the detection of new variants and faster response to control disease spread in the future. In addition, the multiple sequence alignment results showed that there is variable percent of identity between Egyptian isolates from Sharqyiah Province during 2011 and currently used imported vaccinal strains in Egypt .The percent of identity between our isolate and Chinese vaccinal strain was 40.6% and 41% with the Mexican vaccine. while the percent of identity of some reference Egyptian isolates in 2006 with the Mexican vaccine was 78.2% and 92.9% with the Chinese vaccine (Data not shown). This agreed with (Jeong-KiKim *et al.*(2010) who reported the failure of the Volvac vaccine containing A/chicken/Mexico/232/94 (H5N2) to provide complete protection, because this virus is only distantly related antigenically to any of the HPAI H5N1clades. In addition, phylogenetic analysis showed independent sub-clustering of the two viruses (A/chicken/Faquos/amn12/2011(H5N1) and (A/duck/Zagazig/amn13/2011 (H5N1) within the Egyptian sequences that may indicate apossible differential adaptation in the two hosts. This agreed also, with (Madiha *et al.,* 2011) who reported that independent sub-clustering of (A/ck/Egypt/CL6/07) and (A/dk/Egypt D2br10/07) isolated in the same year from chicken and duck respectively, signifying possible differential adaptation in the two hosts.Mass vaccination has failed to control the continuing H5N1 HPAI outbreaks in Egypt. Not only may maternally transferred antibody contribute to this failure, but Standard H5 inactivated vaccines based on phylogenetic distant strains have been widely used in Egypt since 2006. Additionally, backyard poultry that make up an estimated equivalent number of birds to commercial farms are largely not vaccinated. The positive AIV (H5N1) isolates were passaged on different cell lines of avian origin (CEF) and mammalian origin (Vero & MDBK) to determine the differential susceptibility of present isolates on these cell lines. The results showed that the isolate can produce productive infection after addition of trypsin (2µg/ml) after 3 passages on both Vero and MDBK cell lines while directly on CEF. So, CEF,Vero and MDBK cells can be used as alternative systems for AIV isolation but, further studies are needed to determine the best cell line that cannot produce any mutational changes during propagation of viruses to help the authorities for production of cell culture adapted inactivated vaccine from freshly local isolates to control the current outbreaks.

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