MOLECULAR CHARACTERIZATION OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES CIRCULATING IN COMMERCIAL BROILER CHICKENS IN SOME LOCALITIES IN SOHAG PROVINCE

M.A. MOHAMED*, S-E. A. SULTAN***, A.I. AHMED** and A.M. OSMAN****

* Department of Poultry Diseases, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.
** Department of Poultry Diseases, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.
*** Department of Microbiology (Virology), Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.
**** Department of Poultry Diseases, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt.
Corresponding author: moemenassiut@hotmail.com

ABSTRACT

Received at: 25/2/2012

Accepted: 20/3/2012

Thirty one tracheal swabs were collected from diseased broiler chickens experiencing respiratory signs and congestion of head and shank regions and molecularly characterized in Sohag province. The results showed, twenty five (80.6%) of swabs were confirmed positive for carrying type A Avian influenza virus (AIV) by using rapid antigen detection kit. Total RNAs were extracted from the 17 positive AI type A swabs and One step RT-PCR test was performed for subtyping using specific primers targeting H5, H7, N1 and N2. AIV H5 subtype was detected in 9 samples and the rest was untypeable with H7 primers. From the 9 H5 strains, one H5N1 detected and 3 were H5N2 that reflects the presence of two subtypes co-circulated at that time of infection and other untypeable strains with our available primers. Molecular pathotyping was carried on the fully characterized H5 strains using Restriction Enzyme Cleavage Pattern (RECP) assay with the aid of *MboII* restriction enzyme as a new rapid method to avoid the disadvantages of reliable conventional methods (pathogenicity index (IVPI) in specific pathogen free (SPF) chickens and sequencing of hemagglutinin (HA) gene cleavage site). The results revealed that 3 out of 4 (75%) examined H5N1 and H5N2 viruses were pathogenic and one H5N2 strain was low pathogenic. The SDS-page profiling revealed that the examined field isolates were distinct to two clusters; the first one having structural polypeptide: Haemagglutinin (61 KD) and (89KD) protein and the second cluster contains 61 KD, 89KD protein and 50 KDa neuramindase protein. In addition the western blotting showed that the killed Chinese H5N1 vaccine was used in Sohag province by farmers was of low antigenicity which the antibodies reacted only with haemagglutinin protein (61KDa) in all examined strains and one strain reacted with a high molecular weight protein (89KDa). Our data provide proof for the prevalence of H5N2 subtype than H5N1 as well as untypeable strains and low antigenicity of the used vaccine that reflects the

low efficiency of the used vaccine in Sohag. So, the field strains should be updated in a timely manner through surveillance and accompanying laboratory evaluation of contemporary viruses for antigenic similarity with existing vaccine strains and so production of good immunity.

Keywords, Avian influenza, RT-PCR, rapid pathotyping and antigenic analysis

التوصيف الجزيئي لفيروسات أنفلونزا الطيور المتداولة في الدجاج اللاحم التجاري من بعض المواقع في محافظة سوهاج

مؤمن عبد العظيم محمد ، سراج الدين احمد سلطان ، أحمد ابراهيم احمد ، احمد محمد عثمان

تم جمع واحد وثلاثون مسحة قصبة هوائية من الدجاج اللاحم المريض والذى كان يعاني من مشاكل تنفسية واحتقان في مناطق الرأس والساق. أظهرت النتائج أن ٢٥ من المسحات (٨٠,٦٪) إيجابية لحمل فيروس أنفلونزا الطيور (AIV) من النوع (أ) باستخدام عدة الكشف السريع لمستضد فايروس انفلونزا الطيور. تم استخراج RNAs من مجموع ١٧ مسحة ايجابية لفيروس انفلونزا الطيور ثم أنجز اختبار الخطوة الواحدة لتفاعل البلمرة العكسية المتسلسل (One Step RT-PCR) وذلك للتصنيف النوعى لفايروس انفلونزا الطيور باستخدام بادئات تستهدف H5، H7، N2وN1 . تم اكتشاف ٩ من ١٧ عينة تحمل H5 AIV والباقي كان غير متعرف عليه (untypeable) مع بادئات H7 . من الـ٩ سلالات H5 ، كانت واحدة H5N1وثلاث H5N2 الذي يعكس وجود نوعين في ذلك الوقت من العدوى وغيرها من السلالات الغير معرفة untypeable. وللتعرف على ضرواة معزولات H5 تم استخدام طريقة التعرف الجزيئي على الضرواة باستخدام تقييد نمط الانقسام الانزيمى (RECP) باستخدام انزيم *MboII* كأسلوب سريع وجديد لتجنب مساوئ الطرق التقليدية الموثوق بها مثل مؤشر المرضية (IVPI) في الدجاج الخالي من مسببات الأمراض (SPF) وتسلسل موقع الانقسام فى جين تلازن الدموية (HA) ، وأظهرت النتائج أن ٣ من ٤ عترات من H5N1 و H5N2 (٧٥٪) كانت شديدة الإمراض(HPAI) وسلالة واحدة H5N2 كانت منخفضة الإمراض. وكشف التنميط باستخدام-SDS PAGE أن العزلات مجال الفحص كانت مميزة على مجموعتين الأولى تحتوى على: الهيماغلوتينين (٦١ كيلودالتون) و(٨٩ كيلودالتون) بروتين، والمجموعة الثانية تحتوي على ٦١ كيلودالتون ، والبروتين ٨٩ كيلودالتون و ٥٠ كيلودالتون (neuramindase). وبالإضافة إلى ذلك أظهر استخدام الغربي النشاف ان السيرم المحصود من الدجاج المحصن بلقاح H5N1 الصيني المستخدم في محافظة سوهاج من قبل المزارعين منخفض الكفاءة نتيجة لتفاعله فقط فى جميع العينات المفحوصة مع المستضداد البروتينى للهيماغلوتينين (٦١ كيلودالتون) ماعدا فى سلالة واحدة اتحد مع بروتين اضافى عالي الوزن الجزيئي (٨٩ كيلودالتون). من النتائج السابقة نقدم دليلا للانتشار الاكبرلسلالة H5N2 عنها من H5N1 بجانب تواجد انواع اخرى غيرمصنفة مما يفسر انخفاض كفاءة اللقاح المستخدم الى جانب ضعف المستضد البروتينى للتحصين المستخدم. لذلك ينبغي تحديث السلالات المستخدمة فى انتاج اللقاح لتكون معاصرة لسلالات المتداولة والمعزولة من الوبائيات الحادثة.

INTRODUCTION

The first outbreaks of highly pathogenic H5N1 viruses were reported in South East Asia in Guangdong China, since 15 years ago (Tang *et al.*, 1998). Up to date, the virus was reported in more than 60 countries (OIE, 2010).Since then, the disease has become endemic in some Asian and African countries (Poetri *et al.*, 2011). Influenza viruses are members of the family Orthomyxoviridae and are classified as Type A, B and C based on antigenic differences in their nucleoprotein (NP) and matrix (M1) proteins. All avian influenza viruses (AIV) are classified as Type A. Further subtyping is based on antigenicity of the surface glycoproteins viz., haemagglutinin (HA) and neuraminidase (NA). Currently 16 HA and 9 NA subtypes have been identified among influenza A viruses (Fouchier *et al.*, 2005; Palese and Shaw, 2007).

Egypt has experienced outbreaks of H5N1 highly pathogenic avian influenza virus every year since the first detection of virus in February 2006. The estimated loss of the Egyptian poultry industry after the first emergence of highly pathogenic avian influenza (HPAI) H5N1 in February 2006 was 30 million birds were culled or depopulated and 1 billion US$ affected the income of 1.5 million people whose livelihoods depended on poultry (Meleigy, 2007).

The prompt identification of AIVs during a poultry outbreak is critical for a timely control program and reduction of viral spread (Pelzel *et al.*, 2006), any delays in diagnosis or response to an outbreak allow the virus to spread, making eradication increasingly difficult. Identification of AIVs by conventional methods is achieved through isolation of the virus in specific pathogen free (SPF) or AIV antibody negative embryonated chicken eggs followed by HA and NA subtypes identification by the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays, which constitute the standard for AIV subtypes identification (OIE, 2010). The limitations of these antibody-based methods include reagent (antisera and antigen) dependency, cross-reactivity, subjective evaluation, low sensitivity, low specificity, and low accuracy (Hoffmann *et al.*, 2001; Fouchier *et al.*, 2005).

As virus isolation and subtyping are time consuming, many reports over the last decade have demonstrated the use and superiority of PCR-based diagnostic tests in subtyping of influenza viruses within 24 hours especially in the season of outbreaks. So, RT-PCR has been used to distinguish H1–H16, or to differentiate N1- N9 viruses (Stockton *et al.*, 1998; Lee *et al.*, 2001; Poddar, 2002; Kessler *et al.*, 2004).

Based on the pathogenicity of AIVs to domestic poultry, these viruses are subclassified into 2 pathotype groups of Highly Pathogenic Avian Influenza (HPAI) viruses, causing rapid mortality in poultry which approaches100% and Low Pathogenic Avian Influenza (LPAI) causing inapparent diseases with mild respiratory signs, egg production losses and sometimes with slightly elevated mortality (Capua and Alexander, 2006).

To date, all HPAI isolates have been of H5 and H7 subtypes, although viruses of these subtypes do not necessarily cause HPAI (Capua and Alexander, 2006). These viruses are listed as A group diseases by the Office International des Epizooties (OIE) (OIE, 2010). It has posed a potential threat to the health and life of human beings ever since an H5N1 influenza virus was isolated from a 3-year-old boy in Hong Kong, who died of extensive influenza pneumonia complications (Webster *et al.*, 2007). To date, there have been 486 human H5N1 infections, resulting in 287 fatalities, with recent deaths reported in China, Indonesia, Vietnam, and Egypt (WHO, 2010).

AI viruses with an IVPI in 6-week old chickens greater than 1.2 are considered as HPAIV (CEC, 2006). Nevertheless, regardless of their virulence for chickens, the presence of multiple basic amino acids at the HA_0 cleavage site is well established as an accurate indicator of virulence for H5 and H7 influenza viruses (Hoffmann *et al.*, 2007). Therefore, molecular characterization of the cleavage site by sequencing or other methods (Londt *et al.*, 2007) will become the method of choice for assessment of the pathogenicity of these viruses. However, this method requires expensive equipment and consumables, as well as skilled, experienced staff. New molecular methods as restriction enzyme cleavage pattern analysis of RT-PCR products have the advantage of accelerating diagnosis and reducing the risk of handling infectious material (Fereidouni *et al.*, 2009 and Höper *et al.*, 2009) has been used in this study and may be applicable in less well-equipped laboratories.

Vaccination of domestic poultry to prevent AI virus infection is crucial for effective disease control and eradication in animals and for public health as well, but the occurrence of AI outbreaks still occur in Egypt, in spite of variable types of vaccines and regimes of vaccination in commercial and backyard

birds (Hafez *et al.*, 2010). More than 1.3 billion doses of different types of inactivated H5 vaccines mainly based on Eurasian and American H5N2 and Eurasian H5N1 subtype viruses (Aly *et al.*, 2010).

Inadequate protection that was reported may be due to no local vaccines having been prepared and is completely dependent on the use of imported vaccines. For vaccines to have a high efficacy, 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).

The current work was planned to characterize the currently circulating virulent strains of avian influenza viruses (H5 and H7 subtypes) isolates using Reverse Transcription-Polymerase Chain Reaction (RT-PCR), determination the pathogenicity of the isolated strain (s) using the Restriction Enzyme Cleavage Pattern (RECP) as a rapid recent molecular method instead of sequencing of H_0 cleavage site and finally to determine the antigenicity of isolated field and vaccinal strains using western blot assay.

MATERIALS and METHODS

1. Tracheal swabs

Thirty one tracheal swabs were collected from suspected cases of Avian Influenza in different commercial broiler flocks located in Sohag province using sterile cotton swabs and resuspended in 2 ml sterile phosphate buffered saline (pH 7.2), supplemented with penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml). All specimens were transported, chilled at approximately 4°C using ice boxes and delivered to the laboratory within 48 hr. The swab suspension was clarified by centrifugation at 2000 rpm at 4°C for 15 min then the supernatants were filtered through 0.2µm syringe filters (Sartorius, Minisart, Germany) then stored at -80°C till use.

2. Virus detection and identification

2.1. Rapid Antigen Detection Test

Thirty one tracheal swabs were screened by Chromatographic immunoassay test kit for influenza type A antigen detection (Anigen, Korea) according to manufacturer's instructions.

3. Molecular subtyping of the field isolate by one step RT-PCR

- Tracheal swab supernatants were processed for extraction of RNA using Innu PREP RNA Mini Kit (Analytik Jena AG; Jena, Germany) according to the manufacturer's protocol recommendations.

- Each sample was subjected to the detection of AIV H5, H7, and their respective Neuraminidase (NA) subtypes N1 and N2 by RT-PCR. The primers (Alpha DNA, Canada) used for the detection of HA and NA Genes and amplicon sizes are listed in Table 1.

- The RT-PCR was performed using a OneStep RT-PCR kit (QIAGEN INC., Valencia, USA) containing the appropriate primer mix. The reaction components for RT-PCR tube were 13µl RNase-Free water (molecular grade), 5µl (5x Qiagen one step RT- PCR buffer), 1µl dNTP Mix (containing 10 mM of each dNTP), 1µl Primer A (H5-F), 1µl Primer B (H5-R), 1µl Qiagen one step RT-PCR enzyme mix, 3µl Template RNA. The tubes were incubated on ice and then put in the thermal cycler (Techne, Cyclogene thermal cycler. UK.) with the following conditions: Reverse Transcription step at 50° C for 30 min/1cycle, initial PCR activation step at 95° C for 15 min/1cycle, followed by 3-step cycling which include 40 cycles of denaturation step at 94 ° C for 40 sec, annealing at 55 ° C for 40 sec, and extension at 72 ° C for 40 sec. An additional extension step at 72 ° C for 10 min was done to complete the amplification.
- The thermo-profile required for amplifying N1, N2, and H7 were the same conditions of H5 except the amplification of N2 differed only in annealing time was for 1min. Amplified RT-PCR products were electrophoresed on a 1% agarose gel.

Table1: The oligonucleotide sequence of primers used for one step RT-PCR amplification.

Target gene	Primer name	Primer sequence (5'-------3')	RT-PCR product (bp)	References
H5	H5-F	GCC ATT CCA CAA CAT ACA CCC	219	WHO(2002)
	H5-R	CTC CCC TGC TCA TTG CTA TG		
N1	N1-1	TTG CTT GGT CGG CAA GTG C	616	Wright *et al.* (1995)
	N1-2	CCA GTC CAC CCA TTT GGA TCC		
H7	H7-F	TCT CCT TGT GCA TTT TGA TGC	340	Gromadzka *et al.* (2008).
	H7-R	GGG GCT TTC ATA GCT CCA GAT CGT G(		
N2	N2-1	ATG GTC CAG CTC AAG TTG TCA	434	Wright *et al.* (1995)
	N2-2	TCC AGT TAT GTG TGC TCA GG		

4. Rapid pathotyping of the isolates by Restriction Enzyme Cleavage Pattern (RECP) of RT-PCR product

- The extracted RNA of the previously subtyped H5 strains subjected to pathotyping using RECP Fereidouni (2008) as the following:

i- The RT-PCR assay was performed using one step RT-PCR kit (Qiagen, UK) according to the manufacturer's instructions. Reaction components were 11µl RNase-Free water (molecular grade), 5µl (5x Qiagen one step RT- PCR buffer), 1µl dNTP Mix (containing 10 mM of each dNTP), 1µl PrimerA (H5-F; 5'- GAT AAA YTC TAG YAT GCC ATT CC -3'), 1µl PrimerB (H5-R; 5'- TTT TGT CAA TGA TYG AGT TGA CCT TAT TGG-3'), 1µl Qiagen one step RT-PCR enzyme mix, 5µl Template RNA. The tubes were incubated on ice then transferred to Thermocycler with the following cycling conditions; 50° C for 30 min/1cycle (Reverse Transcription), 95° C for 15 min/1cycle (initial PCR activation) followed by 40 cycles of 94 ° C for 30 sec, 50 °C for 45 sec, 72 ° C for 45 sec, and a final extension at 72 ° C for 10 min. Amplicons of sizes between 302 and 314 bp were considered specifically positive.

ii- Restriction enzyme digestion and gel electrophoresis

The resultant specific RT-PCR amplicons were extracted from gel by using agarose gel extraction kit (Jena Bioscience, Germany) according to manufacturer's recommendations. *MboII* restriction enzyme (Fermentas, thermoscientific, USA) was selected to differentiate AIV H5 pathogenicity to low and high pathotypes (Fereidouni, 2008). The optimized reaction mix included 11µl Nuclease-Free water, 3µl 10x fast Digest Green Buffer, 15µl cDNA, and 1 µl *MboII* enzyme. The samples were incubated at 37° C for 10 min and after digestion, at 65° C for 15 min. The resulting cleavage fragments were separated by electrophoresis at 110V for 35 min on 2% agarose gel.

5. Western blot analysis of viral antigens

- To evaluate the specificity of the antibodies raised in response to vaccination against field strains of our study. Eight identified strains were propagated through allantoic inoculation of 9-11 days old embryos, under the Biosafety Cabinet (BSL3) then the eggs were incubated at 37°C for 48 hours where after these were chilled for 4 hours at 4-8°C (Swayne *et al.*, 1998).
- The harvested Allantoic fluid was tested for hemagglutination activity and the positive samples were further analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) through 12% resolving gel. The viral pellets from harvested allantoic fluids were made according to U.S. Pat. No. 3,874,999. The resultant suspension was clarified by low-speed centrifugation and the virus was purified from the resulting supernatant. The samples diluted 1:1 in sample buffer (0.5M Tris HCL; pH 6.8, 20% glycerol, 10% SDS, 0.5% bromophenol blue as tracking dye) was heated in a boiling water bath for 5min

and loaded onto the 12% SDS-PAGE gel, for resolving proteins then analyzed by Western blotting as described below.

- For western blotting, separated proteins were transferred to a nitrocellulose membrane 0.45 μm (Bio-Rad) in a semidry electroblotter (Cleaver; UK) at 2mA/ cm^2 for 2 hour. The protein blot was then treated with blocking solution (PBS containing 1% Tween 20 and 5% nonfat dry milk) overnight at 4°C, followed by incubation for 2 h at 27°C with the primary AI vaccinated chicken sera (dilution 1:8). The membrane was washed three times in blocking solution and reacted with a goat anti-chicken–horseradish peroxidase (HRP) conjugate (Gaithersburg, MD. USA) diluted 1:2000 in blocking buffer for 1 h at RT. one component TMB membrane peroxidase (Gaithersburg, MD. USA) was used for detection of bound HRP conjugate. Molecular weights of the immunogenic viral peptides were determined by comparing their migration fronts to those of the Marker.

RESULTS

Clinical observations

- Infected birds showed facial edema, swollen cyanotic comb and wattles, and diffused hemorrhage in shank region. Subcutaneous edema in the head and neck regions, severe congestion of the musculature Fig. 1(A, B). As shown in Fig. 2(A-C), the necropsy examination of the dead birds revealed organ hemorrhagic lesions on the proventriculus mucosal membrane.

Identification of influenza A viruses

- As shown in Table 1, a total of 25 tracheal samples were developed positive reaction with rapid antigen detection kit (Anigen) out of 31 examined samples (80.6%) where two color bands developed in the reading well Fig.3.

Subtyping of AIV H5/H7 by one step RT-PCR.

- Based on RT-PCR, 9 samples out of 17 tested samples were positive for H5 (52.9%) and produce a specific PCR product at 219bp and the rest of samples were negative Fig. 4. Among the 9 positive H5 samples, one sample was positive for N1 primer (11.1%) and the other 8 samples were negative for N1 as in Fig. 5. This means that only one sample among the 9 H5 positive samples tested was H5N1 subtype. The rest H5 positive samples were tested with N2 primers and the results revealed 3 of 9 were positive to N2 primer (33.3%) Fig. 6 and so there were 3(3/9) samples were recognized as H5N2 (33.3%). This gives indication that H5N2 was more in occurrence than H5N1 at the area of the study.
- The 8 negative samples with H5 primers tested with H7 primers also were negative and no amplicon obtained that indicate there is other circulating AI subtypes present in the area of investigation.

Pathotyping of isolates by RECP of RT-PCR products

The results revealed that the only subtyped strain of H5N1 were highly pathogenic as shown in Fig. 7A in the lane crowned by number 8 which amplicons harbouring a *MboII* recognition site produced two DNA fragments with approximately 130 and 180 bp in size. Also the results showed that 2 out of the three examined H5N2 strains were highly pathogenic as shown in Fig. 7A and B (Lanes crowned by number 9 and 10) in contrast to 1 H5N2 was low pathogenic as seen in Fig. 7A in the lane crowned by no.7 that shown uncut amplified RT-PCR products 310 bp i.e. low pathogenic AIV, did not carry the *MboII* cleavage motif and remained uncleaved.

Antigenic analysis using western blot assay

- Eight different isolates subjected to whole protein profile by SDS-PAGE following electrophoresis, two polypeptides 61kDa which is haemagglutinin protein (HA) and 89 KDa unidentified protein were identified in most of the strains and a 51 KDa protein which is neuraminidase in 5 out of 8 isolates (H5N1), (H5N2), (H5N?), (H5N?), (H5N2), (H5N2) as shown in Fig. 8.

- Also Electrophoretic patterns of proteins analysis mentioned variation between strains and co-circulation of two clusters in the field; the first one contained two proteins (61 and 89 KDa) and the second one contained three proteins (50, 61 and 89 Kda)
- Immunoblotting results revealed the antigeni band of 61 kDa haemaglutinin of HPAI usin polyclonal antibody harvested from vaccinate birds. Furthermore, one isolate (H5N1 expressed additional immunogeni determinant at 89 KD as shown in Fig. 9.

Fig.1 (A): Congestion of the comb and periorbital oedema

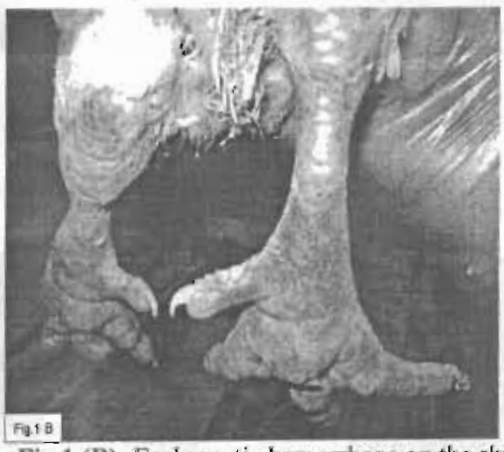

Fig.1 (B): Ecchymotic hemorrhage on the shunk

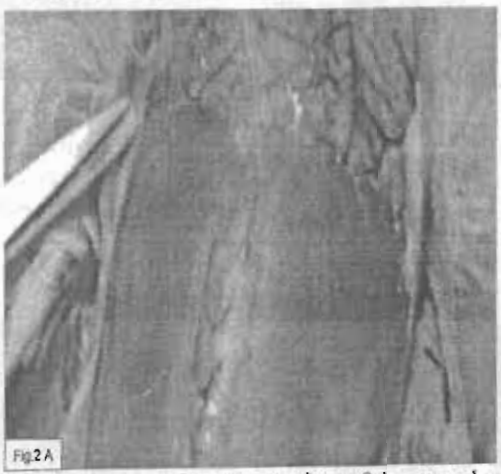

Fig.2 (A): Showing congestion of the muscles. and subcutaneous vessels

Fig.2 (B): Hemorrhages on the junction between proventiculus and gizzard.

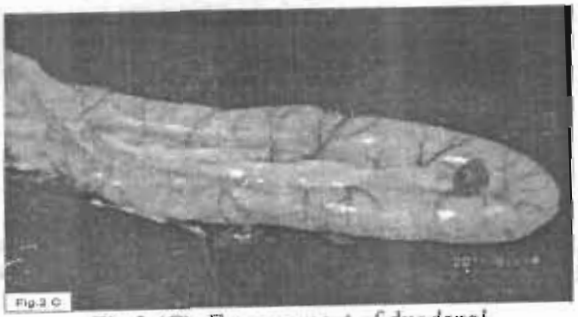

Fig.2 (C): Engorgement of duodenal blood vessels

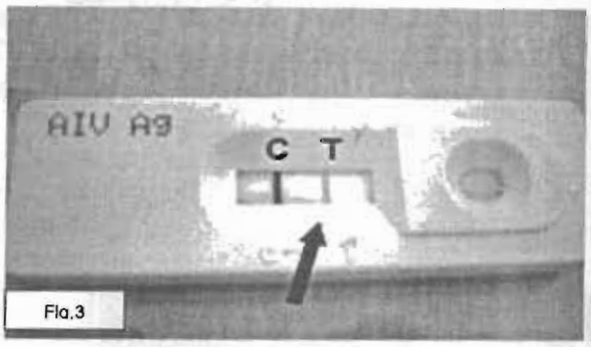

Fig.3: Showing positive reaction of rapid avian influenza antigen test kit

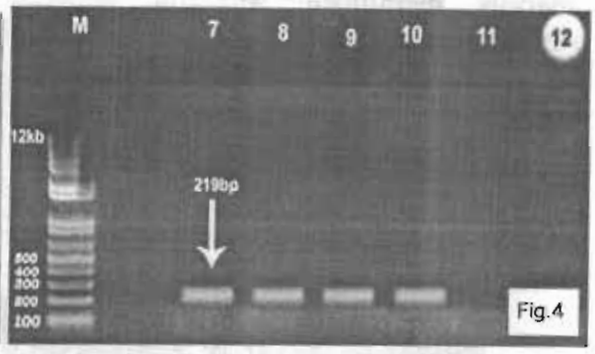

Fig.4: RT-PCR analysis of the subtype specific primer of H5.Lane 7-10 amplicons at 219bp indicating positive reaction. Lane 11-12 negative samples. Lane M: 1Kbplus DNA ladder.

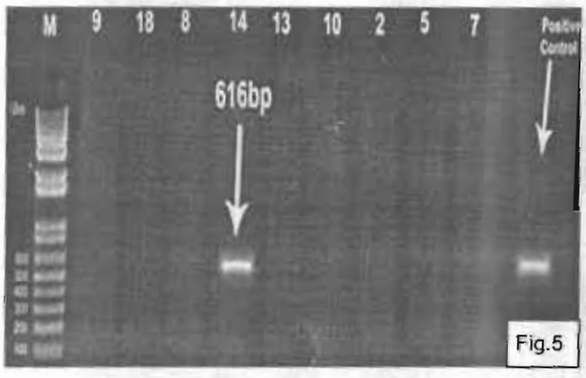

Fig.5. Subtyping by RT-PCR using N1 primer, lane M: 1Kb DNA ladder, Lane 14: positive sample Sample for N1 giving amplicon at 616bp and the same weight of positive control sample.

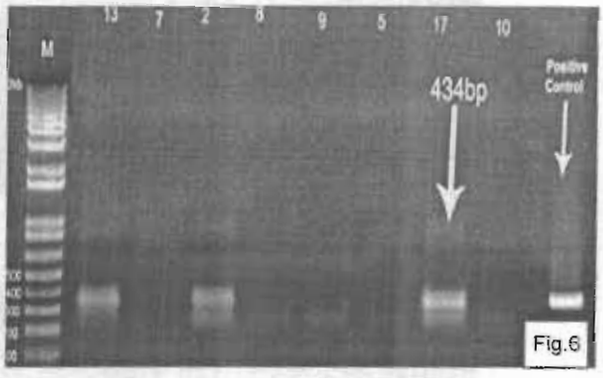

Fig. 6. Subtyping of H5 AIV positive samples by RT-PCR using N2 specific primer, Lane 13, 2, 17 positive lanes for N2 primer giving product at 434bp and compatible with positive control sample.

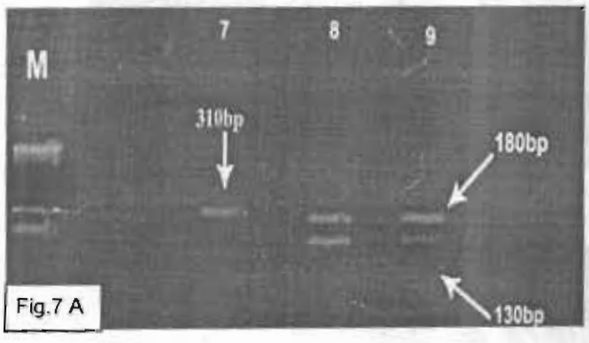

Fig. 7 (A). Gel electrophoresis of H5 RT-PCR products of highly and low pathogenic avian influenza viruses after *MboII* digestion. Lane 7 LPAI amplicon at 302bp while lane 8 and 9 HPAI amplicons at 180bp and 130bp. M: DNA marker

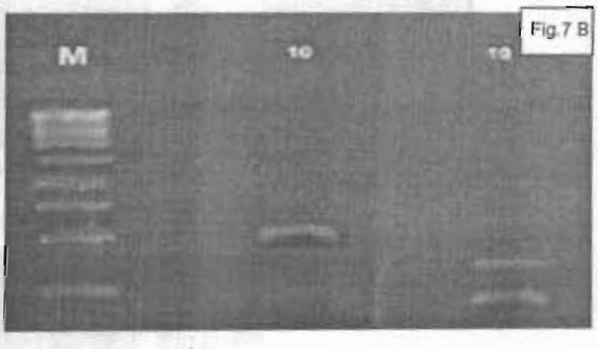

Fig. 7(B). Gel electrophoresis of H5 RT-PCR products before (first lane) and after enzyme digestion (second lane) of the same sample. The enzyme producing two DNA fragments in HPAI.

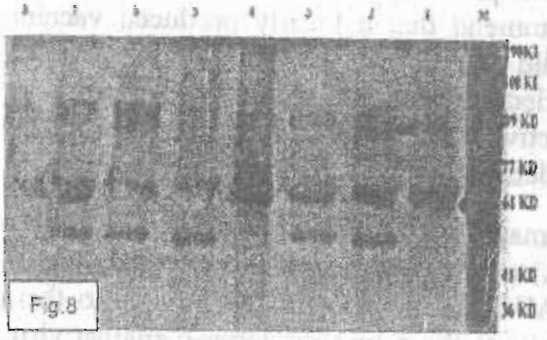

Fig. 8. SDS-PAGE of AIV isolates on the 12% Polyacrylamide with protein marker on the right side of the gel. Lane 1-8 isolates. The isolates. share common protein at 61KD and an unidentified 89KD proteins appear in some isolates.

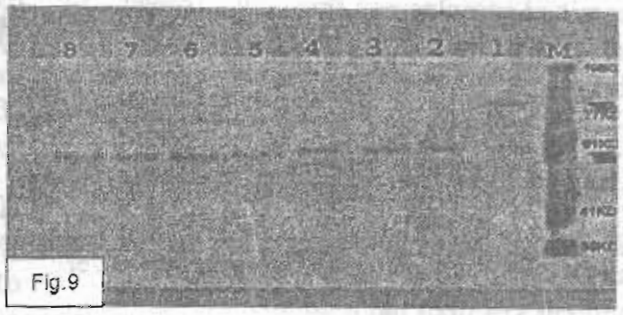

Fig. 9. Western blotting of AIV isolates, M (prestained protein molecular weight marker). Lane 1-8 AIV Isolates showing highly reactive immunogenic epitope at 61 KD expressed in all AIV isolates. One Isolate (Lane 1) expressed additional immunogenic

DISCUSSION

Avian influenza (AI) is a viral disease stretching worldwide in its distribution. Broiler production is the important sector in the Egyptian poultry industry. More than 300 million broilers are bred per year (Abdelwhab and Hafez, 2011). Nevertheless, vaccination of broiler chickens in Egypt, continuous circulation of HPAI H5 virus was reported from chicken flocks despite a history of multiple and variable vaccination regimes (Hafez *et al.*, 2010).

In this study, all the collected tracheal swabs were tested using a rapid influenza A antigen detection kit. Twenty five out of 31 samples (80.65%) were identified as influenza A isolates that confirms the endemic nature of AIV in Sohag province. This level of infection was considered higher if compared with that reported by Mady *et al.* (2010) who recorded 55.6% as a percentage of infection. This result may be attributed to the following; the lack of biosecurity measures in the area of sample collection, percentage of vaccinated flocks, collection of samples either from outbreaks or individual cases, bird species and season of sample collection which the virus isolation was higher in cooler temperatures (Suarez *et al.*, 2008).

Generally, isolation of AIV requires 1–2 weeks for diagnosis virus isolation is dependent on the presence of live virus. Thus, molecular techniques esp. RT-PCR is often used as a rapid screening method for the detection of AIV subtypes with processing of large numbers especially in the avian influenza season and is especially applicable to tracheal and cloacal swabs taken from poultry (Schweiger *et al.*, 2000; Spackman *et al.*, 2002).

Molecular subtyping of Highly Pathogenic Avian Influenza (HPAI) viruses using RT-PCR was recommended (Wright *et al.*, 1995; Stockton *et al.*, 1998). In the present study, a total number of 17 tracheal swabs was tested by RT-PCR and 9/17 (52.9%) samples were H5 which indicated that H5 subtype was the most common. From these 9 samples, one sample was H5N1 as mentioned before by Hafez *et al.* (2010). Also, the results of the present study showed that there are three H5 strains subtyped with N2 primers and H5N2 subtype was predominant than H5N1.

These results in suggest. that the use of H5N2 vaccine in commercial broiler chickens can be more effective in protection than the use of Chinese inactivated H5N1 vaccine which is currently used by farmers in sohag province.

H7 subtype was detected among the examined samples and this result agreed with that mentioned by El-Zoghby *et al.* (2011). This may reflect that there is a new subtype (s) circulating in the field other than H5 or H7 which may explain why vaccination with H5N1 does not provide successful protection against AI which Egypt embarked only on inactivated H5 vaccines to combat the severe outbreaks of high pathogenic AI.

Regarding to the determination of pathogenicity among our fully typed H5 strains, we used a new molecular pathotyping method based on restriction enzyme cleavage patterns (RECP) as mentioned by (Fereidouni *et al.*, 2008). The notion of this technique is depending on the highly pathogenic strains of H5 have a unique nucleotide pattern at the HA_0 cleavage site of hemagglutinin gene at which the restriction enzyme cut. The size of uncut PCR product is 310 bp and when harboring *MboII* recognition site it produced two DNA fragments with approximately 130 and 180 bp in size.

As mentioned in Fig. 7A and 7B the pathotyping of our isolates either H5N1 or H5N2 were of HPAI type except one isolate of H5N2 was LPAI which may approve that there are more than one pathotypes circulated in the field which may be in going to mutate to be a highly pathogenic one under certain stresses as vaccination (Fereidouni *et al.*, 2008).

As shown in Fig. 8, the viral protein analysis by SDS-PAGE revealed that all examined viral isolates have haemagglutinin protein of molecular weight 61KD as recorded by Bahgat *et al.* (2009), neuramindase protein (50KD) is found only in 5 out of 8 examined strains as demonstrated by Bahgat *et al.* (2009) and another protein of high molecular weight (89KD) is not mentioned by any researchers before. Considering the previous results, there is two clusters among examined AI strains; cluster one contained two structural polypeptide 61Kd and 89 KD and the second cluster contains three structural polypeptides (50, 61 and 89KDs). As we mentioned before, there is differences in the protein profiling and so, we can deduce and recommend that a locally produced vaccine should be made from non-variant parts as (nucleoproteins) which may provide an effective and long lasting solution to this pandemic (Shahzad *et al.*, 2008).

Haemagglutinin (HA) and Neuraminidase (NA) are two of the major protective antigens of AIV, and neutralizing antibodies to them constitute the primarily defense against virus infection (Qiao *et al.*, 2003). Functionally distinct roles have been attributed to the humoral response elicited by these two viral proteins. Antibody against the HA generally neutralizes viral infectivity, by interfering either with viral attachment to sialic-acid receptors on the host cell surface or with fusion between viral and endosomal membrane. In contrast, anti-NA antibody does not prevent infection, but can reduce viral replication below a pathogenic threshold so that infection can occur without frank disease (Deroo *et al.*, 1996).

Results of western blotting showed that the killed Chinese H5N1 virus vaccine that used in Sohag was of low antigenicity which the antibodies reacted only with haemagglutinin protein (61KDa) and to another high molecular weight protein (89KDa) for only one strain as shown in fig.9. This finding was not in agreement with Bahgat *et al.* (2009), who observed that the killed Egyptian isolate H5N1 local vaccine was reacted with 4 multiple influenza antigens. Also, as shown in fig. 9 there was an absence for a reaction of harvested serum with the low molecular-weight nonstructural protein-1which is produced in large quantities during infection (Ghedin *et al.*, 2005) which indicated that the used serum in western blotting assay was due to vaccination process not to infection.

In conclusion, the results of the present study suggest the need for further studies to prepare AI killed vaccine from the local isolates (circulating strains).Moreover, monitoring the surveillance and updating laboratory evaluation of contemporary viruses types and antigenic similarity with the existing vaccine strains are highly recommended for better prevention and control of H5N1 and H5N2

associated AI in chicken populations in Egypt.

REFERENCES

Abdelwhab, E.M. and Hafez, H.M. (2011): An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: epidemiology and control challenges. Epidemiol Infect. 139(5): 647-57.

Aly, M.M.; Arafa, A. and Hassan, M.K. (2010): Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. Avian Dis; 52: 269–77.

Bahgat, M.M.; Kutkatb, M.A.; Nasraaa, M.H.; Mostafaa, A.; Webbyc, R.; Bahgat, I.M. and Ali, M.A.A. (2009): Characterization of an avian influenza virus H5N1 Egyptian isolate. Journal of Virological Methods; 159: 144–150.

Capua, I. and Alexander, D.J. (2006): The challenge of avian influenza to the veterinary community. Avian Pathol. 35: 189–205.

CEC, (2006): Council directive 2005/94/EC of December 20, 2005 on community measures for the control of avian influenza and repealing 92/40/EEC.http: //eurlex.europa.eu/lexUriServ/ lexUriSev.do?uri=OJ: l:2006:010:0016: 0065:EN:PDF.

Deroo, T.; Jou, W.M. and Fiers, W. (1996): Recombinant neuraminidase vaccine protects against lethal influenza. Vaccine, 14: 561-569.

El-Zoghby, E.F.; Abdelwhab, E.M.; Arafa, A.; Selim, A.A.; Kholousy, S.G.; Kilany, W.H.; Hassan, M.K.; El-Kanawati, Z.; Aly, M.M. and Hafez, H.M. (2011):* Active surveillance of avian influenza virus in backyard birds in Egypt. The J. of App. Poult. Res. (20)4: 584-588.

Fereidouni, S.R.; Harder, T.C. and Starick, E. (2008): Rapid pathotyping of recent H5N1 highly pathogenic avian influenza viruses and of H5 viruses with low pathogenicity by RT-PCR and restriction enzyme cleavage pattern (RECP). Journal of Virological Methods; 154: 14-19.

Fereidouni, S.R.; Starick, E.; Grund, C.; Globig, A.; Mettenleiter, T.C. and Beer, M. (2009): Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. Veterinary Microbiology; 135(3-4): 253-260.

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

Gromadzka, B.; Smietanka, K.; Dragun, J.; Minta, Z. and Gora-Sochaka, A. (2008): Detection of changes in avian influenza genome fragments by multi-temperature single-strand conformational polymorphism. Journal of molecular and cellular probes; 22: 301-304.

Ghedin, E.; Sengamalay, N.A.; Shumway, M.; Zaborsky, J.; Feldblyum, T.; Subbu, V.; Spiro, D.J.; Sitz, J.; Koo, H. and Other authors (2005): Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* 437: 1162–1166.

Hafez, M.H.; Arafa, A.; Galal, S.; Selim, A.; Hassan, M.K. and Abdelwhab, E.M. (2010): Avian influenza H5N1 infections in vaccinated commercial poultry and backyard birds in Egypt. Poult. Sci. 89: 1609–13.

Hoffmann, B.; Harder, T.; Starick, E.; Depner, K.; Werner, O. and Beer, M. (2007): Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. Journal of Clinical Microbiology; 45: 600-603.

Hoffmann, E.; Krauss, S.; Perez, D.; Webby, R. and Webster, R.G. (2002): Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine; 20:

3165–3170.

Hoffmann, E.; Stech, J.; Guan, Y.; Webster, R.G. and Perez, D.R. (2001): Universal primer set for the full-length amplification of all influenza A viruses. Archives of Virology; 146: 2275–2289.

Höper, D.; Hoffmann, B. and Beer, M. (2009): Simple, Sensitive, and Swift Sequencing of Complete H5N1 Avian Influenza Virus Genomes. J. of Cli. Micr., (47)3: 674–679.

Kessler, N.; Ferraris, O.; Palmer, K.; Marsh, W. and Steel, A. (2004): Use of the DNA flowthru chip, a three-dimensional biochip, for typing and subtyping of influenza viruses. J. Clin. Microbiol. 42 (5): 2173–2185.

Lee, M.S.; Chang, P.C.; Shien, J.H.; Cheng, M.C. and Shieh, H.K. (2001): Identification and subtyping of avian influenza viruses by reverse transcription-PCR. Journal of Virological methods; 97: 13–22.

Londt, B.Z.; Banks, J. and Alexander, D.J. (2007): Highly pathogenic avian influenza viruses with low virulence for chickens in vivo tests. Avian Pathology; 36: 347-350.

Mady, W.H.; EL-Sanousi, A.A.; Amer, H.M.; El-Sabagh, I.M.; Khafagy, A.M. and Saber, M.S. (2010): First year of the highly pathogenic avian influenza H5N1 outbreak in Egypt: Rapid antigenic/molecular diagnosis and virus isolation. International Journal of Virology; 6(2): 73-81.

Meleigy, M. (2007): Egypt battles with avian influenza. Lancet 370:553–554.

OIE. (2010): Update on highly pathogenic avian influenza in animals (type H5 and H7); http://www.oie.int /downld/avian%20influenza/aai-asia.htm.

Palese, P. and Shaw, M.L. (2007): Orthomyxoviridae: the viruses and their replication, in: Knipe, D.M. and Howley, P.M. (eds), fields virology 5th ed. Lippincott Williams and Wilkins, Philadelphia: 1647-1689.

Pelzel, A.M.; McCluskey, B.J. and Scott, A.E. (2006): Review of the highly pathogenic avian influenza outbreak in Texas, 2004. Journal of American Veterinary Medical Association; 228: 1869–1875.

Poddar, S.K. (2002): Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. J Virol Methods 99: 63–70.

Poetri, O.; Bouma, A.; Claassen, I.; Koch, G.; Soejoedono, R.; Stegeman, A. and Van Boven, M. (2011): A single vaccination of commercial broilers does not reduce transmission of H5N1 highly pathogenic avian influenza. Veterinary Research, 42: 74-85.

Qiao, C.L.; Yu, K.Z.; Jiang, Y.P.; Jia, Y.Q.; Tian, G.B.; Liu, M.; Deng, G.H.; Wang, X.R.; Meng, Q.W. and Tang, X.Y. (2003): Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. Avian Pathol.;32 (1): 25-32.

Schweiger, B.; Zadow, I.; Heckler, R.; Timm, H. and Pauli, G. (2000): Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. Journal of Clinical Microbiology; 38: 1552–1558.

Shahzad, M.I.; Naeem, K.; Mukhtar, M. and Khanum, A. (2008): Passive immunization against highly pathogenic Avian Influenza Virus (AIV) strain H7N3 with antiserum generated from viral polypeptides protect poultry birds from lethal viral infection 5: 144-149.

Spackman, E.; Senne, D.A.; Myers, T.J.; Bulaga, L.L. and Garber, L.P. (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.

Stockton, J.; Ellis, J.S.; Saville, M.; Clewley, J.P. and Zambon, M.C. (1998): Multiplex RT-PCR for typing and subtyping influenza respiratory syncytial viruses.

Journal of Clinical Microbiology; 36: 2990-2995.

Suarez, D.L. (2008): Avian influenza. Pages 3–22 in Avian Influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, IA.

Swayne, D.E.; Senne, D.A. and Beard, C.W. (1998): Influenza, In: D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson & W.M. Reed (Eds), Isolation and Identi®cation of Avian Pathogens (4th edn, pp. 150-155). Kennett Square: American Association of Avian Pathologist.

Tang, X.Y.; Tian, G.B. and Zhao, C.S. (1998): Isolation and characterization of prevalent strains of avian influenza viruses in China. Chinese Journal of Animal and Poultry Infection and Disease. 20: 1-5.

US Patent: Williams, G.V. and Kenneth, H. (2009): Virus Production, http://www.faqs.org/patents/app/20090035837

Webster, R.G.; Krauss, S.; Hulse-Post, D. and Sturm-Ramirez, K. (2007): Evolution of Influenza A Viruses in Wild Birds. Journal of Wildlife Diseases, 43(3): S1–S6.

WHO (2002): World Health Organization. WHO manual on animal influenza diagnosis and surveillance. World Health Organization, Geneva.

WHO (2010): World Health Organization. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO as of 12 March 2010. World Health Organization.Available: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_03_12/en/index.html.

Wright, K.E.; Wilson, G.A.; Novosad, D.; Dimock, C.; Tan, D. and Weber, J.M. (1995): Typing and subtyping of influenza viruses in clinical samples by RT-PCR. Journal of Clinical Microbiology; 33: 1180-1184.