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Antigenic and Genetic Characterization of Avian Influenza H5N1 Subclade 2.2.1/C in Egypt During 2014

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Abstract: Continuous evolution with evolving of different H5N1 sublineages in Egypt put an urgent need for understanding the evolutionary dynamic of H5N1 AIVs which will help for improving the control measures. Egypt was officially endemic with H5N1 AIVs since 2008. In the present study, twenty four tracheal samples were collected from clinically infected flocks suspected to be infected with H5N1 AIVs from different Egyptian Governorates during 2014 for antigenic and genetic characterization. There are two genetic subclades of H5N1 AIV have been circulated in the Egyptian poultry sectors which were belonging to clade 2.2.1. Continuous circulation of classical group 2.2.1/C of avian influenza viruses in the Egyptian poultry sectors was reported in this study. However, this study report the infection of poultry flock with the classical group in spite of different vaccination regimes based on RT-PCR, sequencing and phylogenetic analysis for partial HA gene. More studies and surveillance are needed for better understanding mechanisms of H5N1 AI transmission and reducing the risks of possible future influenza pandemic beside other control measures.

Key words: Evolution · Dynamic · Subclades · Escape mutant · Classical · Risks · Control

INTRODUCTION

Avian influenza was first identified as a distinct disease of poultry in 1878 [1]. Endemic infections or sporadic outbreaks of avian influenza have been reported in poultry populations globally since 1990. Therefore, infected human cases with HPAI H5N1 were reported in Hong Kong during 1997 and known as "bird flu". Over the next 10 years, H5N1 HPAI in poultry spread across Asia, Europe and Africa. In addition, the continued occurrence of zoonotic infections has created a public health crisis with concerns that this virus potentially could reassort with a human influenza A virus to produce the next human influenza pandemic [1, 2].

Avian Influenza virus belongs to Orthomyxoviridae family of negative-sense segmented RNA viruses that are divided into five different genera, including influenza type A, B and C, Isavirus and Thogotovirus. Influenza viruses type A are the most widespread and important members of the family infecting many different avian and mammalian species [3]. The species jumping ability of IVs can result in the infections of poultry and mammals, such as chicken, swine, equine or whales with different virulence levels [4]. To date, ten different genetic clades (0 – 9) of H5N1 virus have been distinguished which further diversified into subclades [5]. There are at least 18 HA subtypes and 11 NA subtypes, designated H1-H18 and N1-N11 respectively [6, 7].

Endemic status for avian influenza H5N1 in Egypt was officially reported since 2008 while the first outbreak from this virus was reported since February 2006 [8]. Phylogenetic analysis for Egyptian HPAI H5N1 viruses revealed 2 distinct groups of viruses co-circulating in poultry; variant and classical groups [9- 12]. Some reports suggest that one of these groups (classical group) is genetically similar to the initially introduced H5N1 viruses to Egypt during 2006; this group was the prevalent among backyard flocks and responsible for human cases [9, 13]. The other group (variant group) arose from a variant strain that emerged during 2008; this group is prevalent

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among vaccinated commercial poultry farms, indicating that the original variant may have been a vaccine-escape mutant [9, 10]. Influenza viruses in a new host revealed a high and predictable evolution level as a result of high error rate of the virus and host selection pressures [14]. The objective of this study is the isolation and molecular characterization for five Egyptian H5N1 AIVs collected from clinically infected vaccinated commercial flocks during 2014.

MATERIALS AND METHODS

Samples: Specimens were collected from different Egyptian Governorates and transported in a suitable transport medium in ice. The collected Specimens for virus isolation were chilled in an ice pack immediately after collection. The specimens were kept frozen at or below -70°C.

Virus Isolation: Tracheal swabs were collected into 1-2 ml of phosphate buffer solution (PBS) containing antibiotics, then filtered through 0.45 µm syringe filter. SPF embryonated chicken eggs were obtained from SPF production farm (Koum Oshiem, Fayoum, Egypt). ECEs were incubated in the egg incubator at 37°C with humidity 40-60% till the age of 9-11 day old and were used for virus inoculation and isolation. The prepared inoculums were inoculated via allantoic sac using 0.1 ml as inoculum/egg. Inoculated eggs were incubated at 37°C for 5 days with daily candling for mortalities investigations. The allantoic fluid was harvested and subjected to haemagglutination activity by rapid slide haemagglutination (HA).

Haemagglutination (HA) and Haemagglutination Inhibition (HI) Assays: Harvested allantoic fluid is collected in sterile tube and make slide haemagglutination test for rapid detection of haemagglutination activity. If slide HA was found positive, the standard quantitative HA plate test will be conducted [15]. For further identifications, the haemagglutination inhibition test is carried out by using reference H5N1 antiserum against by standard protocol [15]. The HI titer is the highest dilution of antigen causing complete inhibition of H5 avian influenza antiserum. The HI unit of each isolates considered the last dilution of antigen giving haemagglutination inhibition.

Oligonucleotides: A set of primers were designed to amplify a region of haemagglutinin (HA) gene of H5N1 AIV. The primer sequences include: H5F: 5'-CCTCCAGARTATGCMTAYAAA -3' and H5R: 5'-TACCAACCGTCTACCATKCCY -3' to amplify 320 bp, fragment of HA1 of HA gene.

Viral RNA Extraction and RT-PCR Assay: RNA was extracted from sample pools using Trizol LS® reagent (INVITROGEN, Carlsbad, CA, U.S.A) according to the manufacturer's instructions. One-step RT-PCR was performed using the verso one step RT-PCR kit (Thermo, USA) according to the manufacturer's instructions.

Thermal cycling conditions for amplification of partial HA gene started by: Reverse transcription at 50°C for 15 min, followed by an initial denaturation step at 95°C for 2 min. cDNA was then amplified with 40 cycles of 95°C for 30 seconds, annealing at 54°C for 1 minute and extension at 72°C for 1 minute. A final extension step was at 72°C for 10 min. The amplified product (5µl) were loaded onto 1.5% agarose gel containing 0.5 µg/ml ethidium bromide for nucleic acid visualization. Electrophoresis was conducted using 1x TAE buffer and PCR products were visualized under UV trans-illumination.

Sequencing and Sequence Analysis: Gene sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an ABI PRISM® 3100. Comparative analysis of HA gene sequences of the H5N1 viruses was carried out and compared with the available sequences using the National Center for Biotechnology Information (NCBI) influenza virus resources database for avian H5N1 viruses.

Phylogenetic analysis of the newly obtained nucleotide sequences in this study was carried out using MEGA 5 software. The evolutionary history was inferred using the neighbour-joining method and the reliability of each tree branch was estimated by performing 1000 bootstrap replicates [17].

Sequence Submission to Gen Bank: The obtained sequence was submitted to NCBI GenBank by following instruction of the BankIt tool of the GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank

RESULTS

Clinical Signs: The affected birds suffered from respiratory distress in the form of coughing, sneezing, rales associated with nervous manifestations, whitish and greenish diarrhea, cyanosis of comb, wattles as well as subcutaneous hemorrhages in the shank (Fig. 1).

Fig. 1: Clinical signs and postmortem lesions of clinically infected chickens with H5N1 avian influenza virus

Fig. 2: Severe embryonic congestion with hemorrhage as aresult of H5N1 AIV propagation in ECEs

Post Mortem Lesions: Severe congestion and subcutaneous hemorrhages for pectoral and thigh muscles were observed. Enlargement associated with congestion for liver, spleen and pancreas. The kidney was severely enlarged and pale with urate depositions in the ureters. The cardiac blood vessels were obviously engorged with blood (data not shown).

Isolation and Propagation: Daily observation for the inoculated ECEs was carried out to investigate the embryo viability. The embryos were died within 48 – 72 hr after inoculation revealing diffuse hemorrhagic lesions (Fig. 2). The allantoic fluid was harvested and tested for HA activity according to OIE Manual [15] recording titers ranged from 5 log 2 to 7 log 2 according to the concentration of the virus in the collected allantoic fluids.

Haemagglutination and Haemagglutination Inhibition Assays: Antigenic characterization for H5N1 by HA assays revealed high titer with agglutination of RBCs. HI assay with reference H5N1 AIV was conducted which revealed inhibition for the haemagglutination biological propriety of H5N1 AIV (Data not shown).

Sequence Analysis: The obtained sequences were subjected to Blast tool of GenBank (NCBI) for further identify the homology percentages and relationships with the co-circulating H5N1 AIVs. Multiple nucleotide alignment revealed the high similarity with the Egyptian H5N1 AIVs (Fig. 3). The polybasic cleavage site is the prime virulence determinant for highly pathogenic avian influenza viruses (HP AIVs) [18, 19].

Deduced amino acid analysis were focusing on the cleavage site of haemagglutinin (HA) gene for the obtained sequences which revealed difference of the cleavage site sequence between the isolates under study (Fig. 4). There are characteristic motifs; the first one was EKRRKKRG for subclade 2.2.1/C H5N1 in the five isolated strains as well as the second motif was EGRRKKRG for variant subclade 2.2.1.1 H5N1 AIVs not observed in our study [20]. These results indicate the circulation of subclade 2.2.1/C of H5N1 AIV in the commercial poultry sectors during 2014 (Fig. 4).

Phylogenetic Analysis: Phylogenetic analysis for the obtained sequences was carried out to further identify the genetic spectrum and evolution of H5N1 AIVs. As previous published data, there are two major genetic groups of highly pathogenic avian influenza H5N1 AIVs have been circulating among the Egyptian poultry sectors [20]. In this study, we reported the continuous circulation of subclade 2.2.1/C (classic) among the poultry population

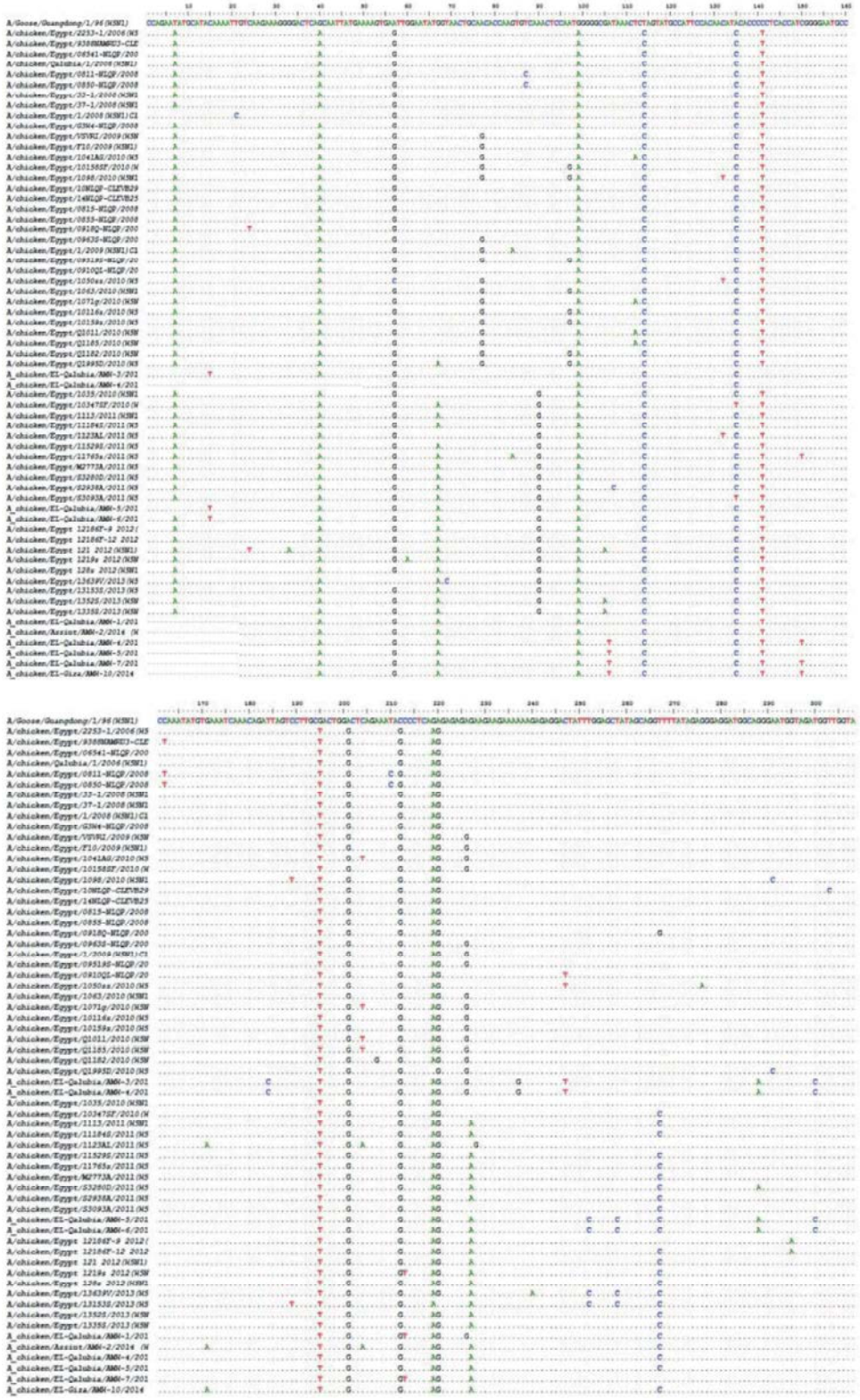

Fig. 3: Multiple nucleotide sequences alignment of partial HA gene of our circulating Egyptian strains during 2014 in comparison with other representative strains of H5N1 AIVs circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence

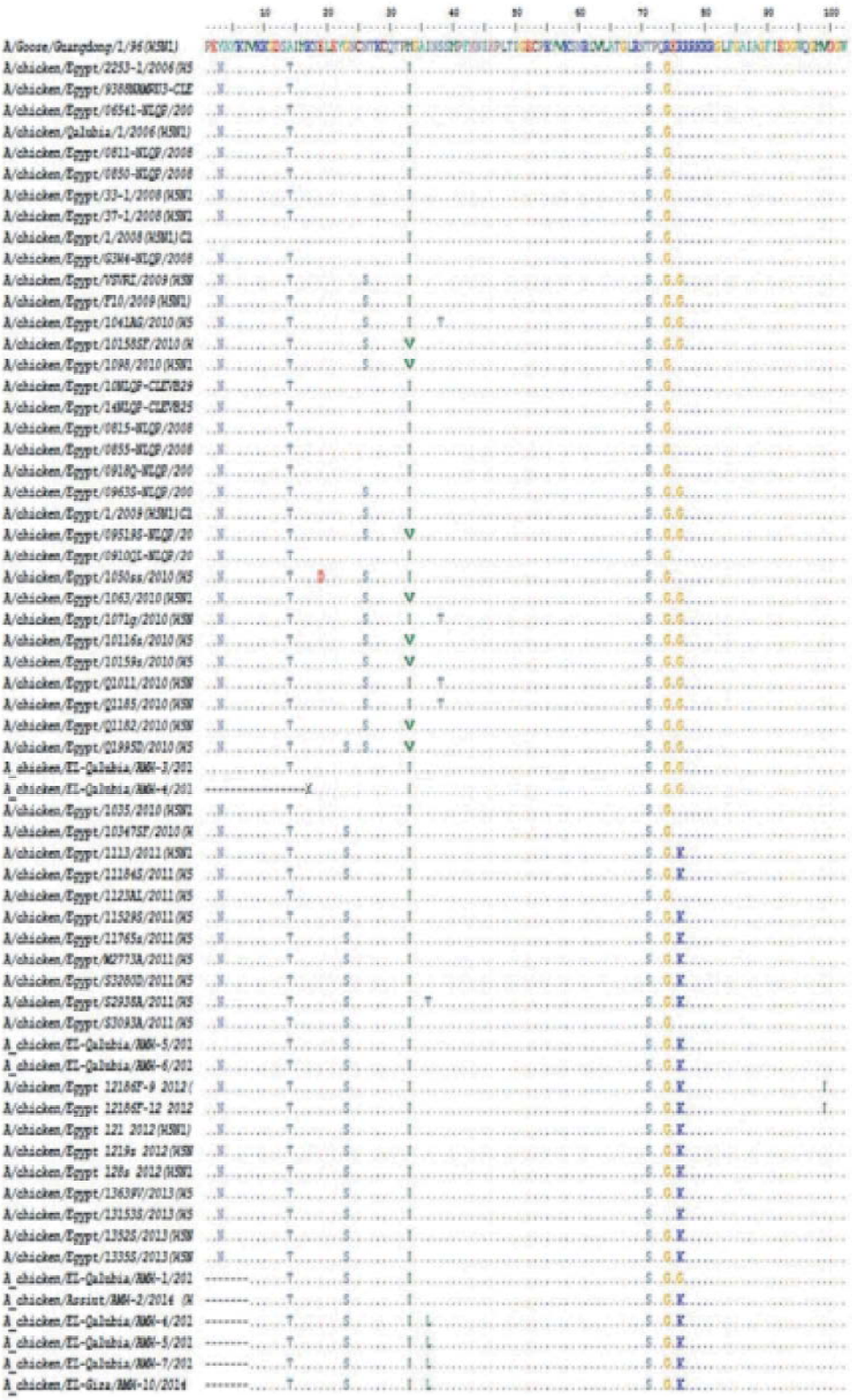

Fig. 4: Amino acid alignment of HA gene of our Egyptian H5N1 AIVs in comparison with other representative strains of H5N1 AIVs circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence

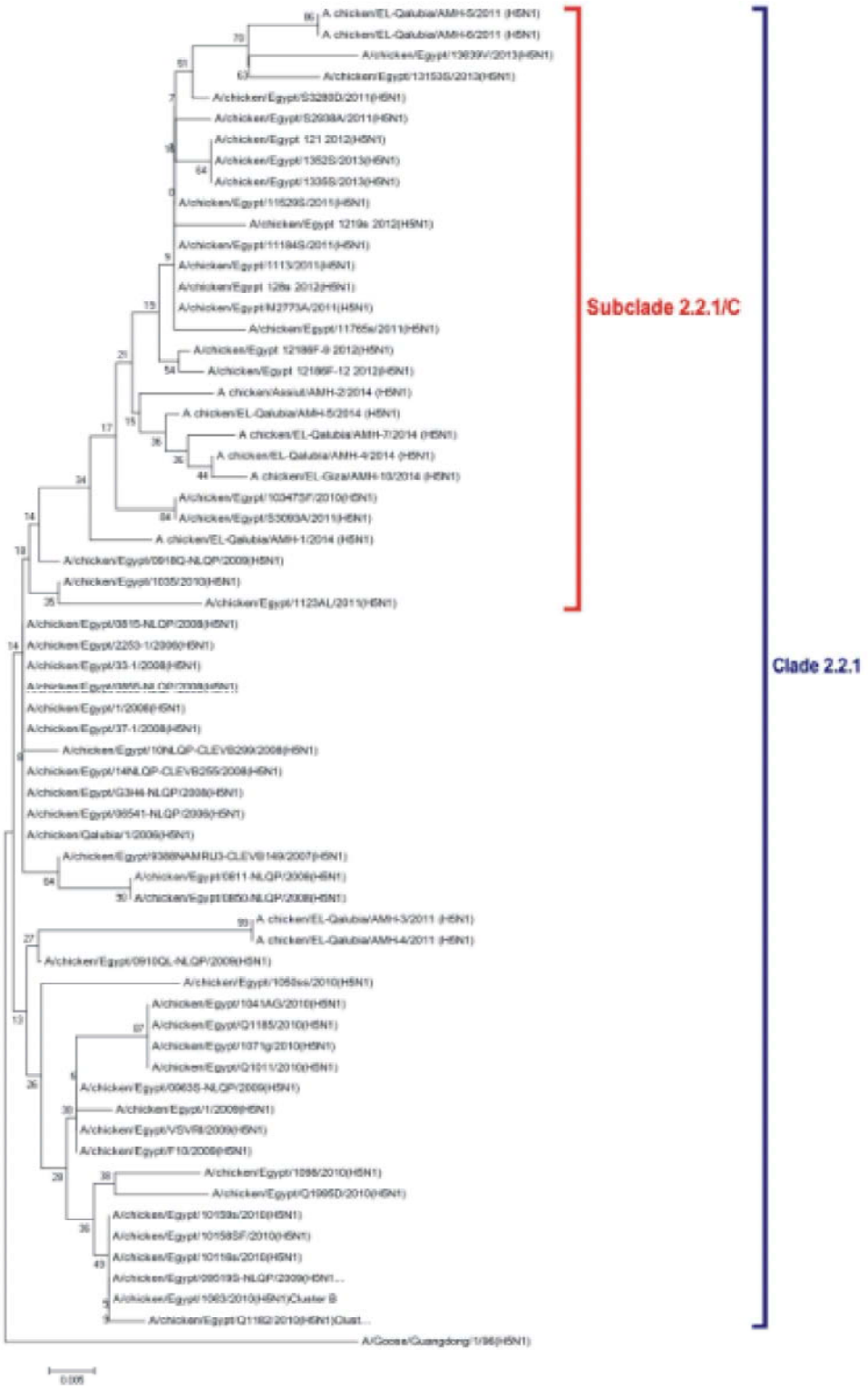

Fig. 5: Neighbor joining Phylogenetic rooted tree with (A/Goose/Guangdong/1/96 (H5N1)) for partial HA gene based on nucleotide sequence showing the clustering of five Egyptian H5N1 AIVs within the classical subclade 2.2.1/C while no isolates clustered within the variant subclade 2.2.1.1 during 2014 season in comparison with other representative H5N1 AIVs. The tree was generated by Mega4 software program

in Egypt during 2014 (Fig. 5) indicating the progressive evolutionary rate for influenza viruses in Egypt which acquired new mutations every season which may lead to adaptation of the virus from avian to mammalian receptors especially that subclade clustered within the same avian influenza viruses isolated from mammalian host and shared some amino acids.

Submission to Gen Bank: After submission of the obtained sequences to Gen Bank, accession number were received and were as follow KM488413, KM488415, KM488416, KM488417 and KM488418.

DISCUSSSION

Influenza A viruses are very important veterinary and human health pathogens around the world [22]. Highly pathogenic avian influenza virus H5N1 (HPAI-H5N1) has disseminated widely across Asia, Europe and Africa affecting a wide range of domestic and wild avian species and sporadically infecting humans and other mammals [23].

Egyptian H5N1 viruses were exclusively classified as clade 2.2.1, which were further subdivided into two major groups; group A (classic subclade 2.2.1/C) and group B (variant subclade 2.2.1.1) [9]. During the last five years, continuous replication of H5N1 virus in Egypt has provided an urgent need to study the relationship between genetic evolution and selection of influenza virus phenotypes. However, mass vaccination has failed to control H5N1 HPAI outbreaks in Egypt [24]. However, variant H5N1 strains were detected in several vaccinated farms in Egypt during 2007 [25]. Rapid divergence and high evolution rate of avian influenza H5N1 accelerating the importance of surveillance and availability of epidemiological data which will help for planning an effective control strategy.

The epidemiological surveillance may be carried out based on serological and or molecular detection. Serological detection for viruses might be not suitable now especially during emergency cases and epidemic viruses. Molecular detection is nowadays is a valuable way for confirmatory diagnosis. In the present study, molecular characterization for suspected infected samples with H5N1 AIV based on RT-PCR, sequencing and phylogenetic analysis. RT-PCR was carried out by using specific primers flanking the cleavage site of haemagglutinin (HA) gene of H5N1 AIV. Twenty four tracheal swabs were collected from commercial poultry flocks during 2014 suspected to be infected with avian influenza H5N1 showing characteristic clinical signs and post mortem lesions.

From the molecular point of view the difference between low pathogenic (LP) viruses and high pathogenic (HP) influenza viruses can be as small as a single amino acid change in the haemagglutinin cleavage site [22]. The polybasic cleavage site is the prime virulence determinant for highly pathogenic avian influenza viruses (HPAIVs) [18, 19]. However, transformation of the polybasic cleavage site to a monobasic motif results in a drastic reduction in virulence [26], conversion of the monobasic HA cleavage site of LPAIVs to a polybasic motif may or may not lead to HPAIVs [27, 28, 29]. Identity percentage for isolates under study was ranged between 99-100% within the circulating geographically related strains among the Egyptian poultry sectors. Point mutations and allelic combinations possess a crucial effect on the virulence of HPAI H5N1 isolates and are thought to be polygenic [30]. During 2010/2011 season, epidemiological surveillance data revealed that the classic group was prevailing in household and backyard birds while the variant viruses were prevailing in commercial vaccinated farms while in this study we reported the co-circulation of both classic and variant subclade in vaccinated commercial flocks [21].

Multiple nucleotide alignment on the obtained sequences for further identifies the nucleotide substitution sites which reflect on the amino acid residues was carried out. Deduced amino acids analysis revealed that difference at the cleavage site of HA gene between classic and variant subclade H5N1 AIV. Some previous studies reported that the EKRRKKR/G motif is characteristic for classic subclade 2.2.1/C as well as ER/GRRKKR/G motif is characteristic for variant subclade 2.2.1.1 [20] which confirmed in this study. Phylogenetic analysis revealed that there are two major genetic groups of H5N1 AIVs are co-circulating in the Egyptian vaccinated commercial poultry sectors since 2007 with the continuous evolution for the classical subclade 2.2.1/C during 2014.

Continuous evolution of H5N1 viruses and the emergence of new variants may be partially explained by immune pressure caused by the use of vaccines, which such variants can circumnavigate vaccine-induced immunity and are called escape mutants [31]. Rapid evolution rate and occurrence of antigenic drifts at the antigenic sites of HA gene may favor the emergence of new sub lineages and or potential pandemic. H5N1 AIVs isolates from human cases are clustered within the avian H5N1 AIVs that may allow avian to human transmission.

Large-scale surveillance of avian influenza viruses H5N1 in endemic areas should be expanded to better understand evolution of influenza viruses and In conclusion, circulation of HPAIV H5N1 in vaccinated birds continues to devastate the poultry industry in Egypt.

CONCLUSION

Continuous circulation of the classical subclade 2.2.1/C of avian influenza H5N1 among the poultry sectors was reported during 2014 with characteristic motif at the cleavage site resembling that isolates from human cases. These results indicate the adaptation of avian influenza viruses in both avian and mammalian hosts. Sustained sequences comparison with phylogenetic analysis is necessary to recognize newly emerging influenza variants and to monitor the global spread of these viruses.

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