Global Veterinaria 14 (5): 662-669, 2015
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2015.14.05.94168

Genetic Diversity of Avian Influenza H5N1 Subclade 2.2.1/C in Commercial Poultry in Egypt During 2013

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Abstract: Two major genetic distinct groups of H5N1 viruses have been characterized in the commercial poultry sectors in Egypt; the classic group of subclades 2.2.1 and the variant subclade 2.2.1.1. The classic group was mainly isolated from backyard birds in 2006 and isolated from all human death cases. Since 2009-2010, this classical group has characterized in commercial sectors in low percentage and became the most prevalent by the end of 2012 with prominent mutations compared to the originally introduced H5N1 AI. Extensive circulation of the classical viruses in vaccinated birds with occurrence of mutations events generated a phylogenetically distinct new subgroup denoted as cluster C. In the present study, we describe the genetic diversity of six Egyptian H5N1 viruses collected from different commercial poultry sectors during 2013 based on genetic analysis and its reflection on the genetic diversity of H5N1 AIVs. It was found that viruses of cluster C shared several amino acid mutations in the HA globular head and retained the classical binding preference for α 2, 3-linked sialylglycan. Continous monitoring of the evolving H5N1 virus in Egypt is essential to develop a new control strategy due to progressive adaptation of the virus in Egypt.

Key words: Cluster · Circulation · Mutation · Evolving · Adaptation

INTRODUCTION

Influenza A viruses are belonging to Orthomyxoviridae family; these viruses havean RNA genome consisting of eight gene segments [1]. Highly pathogenic avian influenza virus H5N1 (HPAI-H5N1) has been disseminated widely across Asia, Europe and Africa affecting a wide range of domestic and wild avian species and sporadically affect humans and other mammals since its emergence in 1996 in Guangdong, China [2,3]. The species jumping ability of influenza viruses can result in the infections of poultry and mammals with different virulence levels [4, 5].

To date, there are ten different genetic clades (0-9) of H5N1 virus have beencirculated which further diversified into subclades [6]. There are at least 18 haemagglutinin (HA) subtypes and 11 neuraminidase (NA) subtypes, designated as H1-H18 and N1-N11 respectively [7, 8]. There are pronounced genetic variation of the surface glycoproteins HA and NA of influenza A viruses. Consequently, classification of influenza viruses was carried out based on the antigenic variation of the HA and NA proteins [9]. Wild birds are considered the natural reservoir of influenza viruses in which over 118 combinations of IVs can be isolated except H17N10 and H18N11 [7, 8]. HA gene is responsible for binding to sialic acid receptors on host cells and for fusion between the viral and host cell membranes upon endocytosis [10].

The first outbreak of avian influenza H5N1 in poultry in Egypt was reported officially in February 2006 [11]. Extensive circulation with persistence of clade 2.2. HPAI H5N1 viruses has led to the spread of infection among domesticated birds throughout the country. HPAI H5N1 virus was declared endemic in Egypt in July 2008 [11]. The Egyptian H5N1 viruses continue to mutate and rapidly evolve over time [12, 13]. During the last 5 years, continuous evolution of H5N1 virus in the Egyptian poultryhas provided an urgent need to study the relationship between genetic evolution and selection of influenza virus strains including antigenicity, receptor binding specificity andpathogenicity. Few reports described the effect of genetic evolution of influenza viruses on their biological characters as well as previous studies focused only on the genetic evolution [14].

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During the H5N1 epidemic in Egypt, several commercial inactivated H5 vaccines using different H5 virus strains were used [15]. Control of HPAI H5N1 outbreaks in Egypt has been failed by mass vaccination [16]. The vaccination campaign limited the first wave of 2006 outbreaks. However, antigenic variants were detected in several vaccinated farms in 2007 and are now the dominant strains in vaccinated and non- vaccinated flocks in Egypt [17]. There are conflicting data on whether commercially available vaccines provide protection against the new antigenic variant strains. Inadequate protection has been reported in some studies [15, 17, 18] while sufficient protection of others have been reported [19, 20]. The major antigenic drift of H5N1 AIVs generated a phylogenetically distinct cluster from the classic subclade 2.2.1/C [21].

In the present study, we describe the evolutionary pattern of Egyptian H5N1 viruses based on molecular characterization of partial H5 gene of six isolates collected from commercial poultry farms during 2013 for identifying the amino acid mutation sites with phylogenetic analysis of partial HA gene to establish the genetic spectrum of these viruses.

MATERIALS AND METHODS

Virus: Tracheal samples were collected from suspected or clinically infected flocks associated with high mortality rates from different governorates in Egypt during 2013 showing characteristic clinical signs and pathognomic postmortem lesions (Table 1).

Table 1: History of suspected collected field samples

Species	Governorate	Year	Host	Sample Type
Chicken	El- Menofia	15-Jun-2013	Broiler breeders	Trachea
	El-Giza	20-Jun-2013	Commercial layers	Trachea
	El- Menofia	17-Feb-2013	Commercial broilers	Trachea
	El-Sharqia	May-2013	Commercial layers	Trachea
	El-Giza	August-2013	Layer breeders	Trachea
	El-Giza	December-2013	Commercial broilers	Trachea

*The six flocks were vaccinated.

*The morbidity rate was 100%.

*The mortality rate was ranged from 85:95%

Oligonucleotides: Sequence data for the hemagglutinin (HA) gene of H5N1 AIV were obtained from GenBank and aligned using the BioEdit software version 7. 0. 4. 1 [22]. Primers that amplify a region of HA gene of H5N1 AIV were designed as follows: VH5F: GAT TGT AGT GTA GCY GGA TGG and VH5R: CTT GTC TGC TCT KCM KCA TC to amplify 406 bp.

Viral RNA Extraction and RT-PCR Assay: RNA was extracted from a tracheal homogenate using Trizol LS® reagent (Invitrogen, Carlsbad, CA, UK) according to the manufacturer's instructions. One-step RT-PCR was performed using the verso one step RT-PCR kit (Thermo, UK). RT-PCR was performed in 25-ul reaction volumes containing 12.5 µl of the 2 X thermo RT-mastermix, 1.25 µl enhancer, 0.5 µl Reverse Transcriptase (RT) enzyme, 0.5 µl of each primer (10 pmol of each primer) and 5 µl extracted RNA. Primers were used at a final concentration of 20 µM/µl, the final volume was reached by adding 3. 75 µlRNase- freewater.

Thermal cycling profile was consisting of: reverse transcription at 50°C for 15 min, followed by an initial denaturation step at 95°C for 2 min. cDNAs were then amplified with 40 cycles of 95°C for 30 seconds, annealing at 51°C for 1 minute andextension at 72°C for 1 minute, followed by a final extension step at 72°C for 10 min. The amplified product (5µl) were loaded onto 1. 5% agarose gel containing 0. 5 µg/mlethidium 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: PCR products were purified separately from gels using QIAEX® Gel Extraction Kit (Qiagen Sciences, Maryland,USA). Purified PCR products were sequenced in both orientations by the dideoxy chain-termination method using the amplification primers which described above. Gene sequencing was carried out using a BigDye Terminator v3. 1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an ABI PRISM®3100. BioEdit software version 7. 0. 4. 1 [22] was used to analyze and assemble the generated nucleotide sequences of the HA gene. The GenBank database was screened (BLAST) for closely related sequences. 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 studyusing MEGA 4. 0. 2 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 [23].

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

RESULTS

Nucleotide Sequence Analysis of Partial HA Gene: To further identify the genetic characteristics of Egyptian H5N1, partial nucleotide sequence of HA gene was carried out and compared with other representative circulating Egyptian strains among poultry sectors and human population which are available on GenBank. Nucleotide Identity percentages with representative H5N1 avian and isolates from human cases were ranged between 92% and 99% which indicate the extensive genetic diversity and evolution for the circulating H5N1 Egyptian strains. Multiple nucleotides revealed extensive nucleotide substitutions at different siteson HA gene especially HA1 part which has the main antigenic sites (Fig. 1).

Mutation Trend Analysis of Partial HA Gene: Multiple amino acids alignment revealed the presence of characteristic deletion of receptor binding domain (Δ129S) (Fig. 2) according to H5 numbering [24].

Phylogenetic Analysis: A phylogenetic tree was constructed for better understanding the genetic relatedness and evolution of Egyptian H5N1 strains. The Egyptian H5N1 viruses were belonging to clade 2.2.1 of HPAI H5N1 viruses. Two major genetic distinct groups of H5N1 viruses have been characterized in the commercial poultry sectors in Egypt; the classic group of subclade 2.2.1 (cluster A) and the variant group 2.2.1.1 (cluster B). Adistinct new subgroup (denoted as cluster C) was diversified from the classical group butdiffer away in the evolution rate which become the predominant phenotype circulated among the vaccinated and non-vaccinated Egyptian poultry populations with clustering within the H5N1 AI isolated from human cases (Fig. 3).

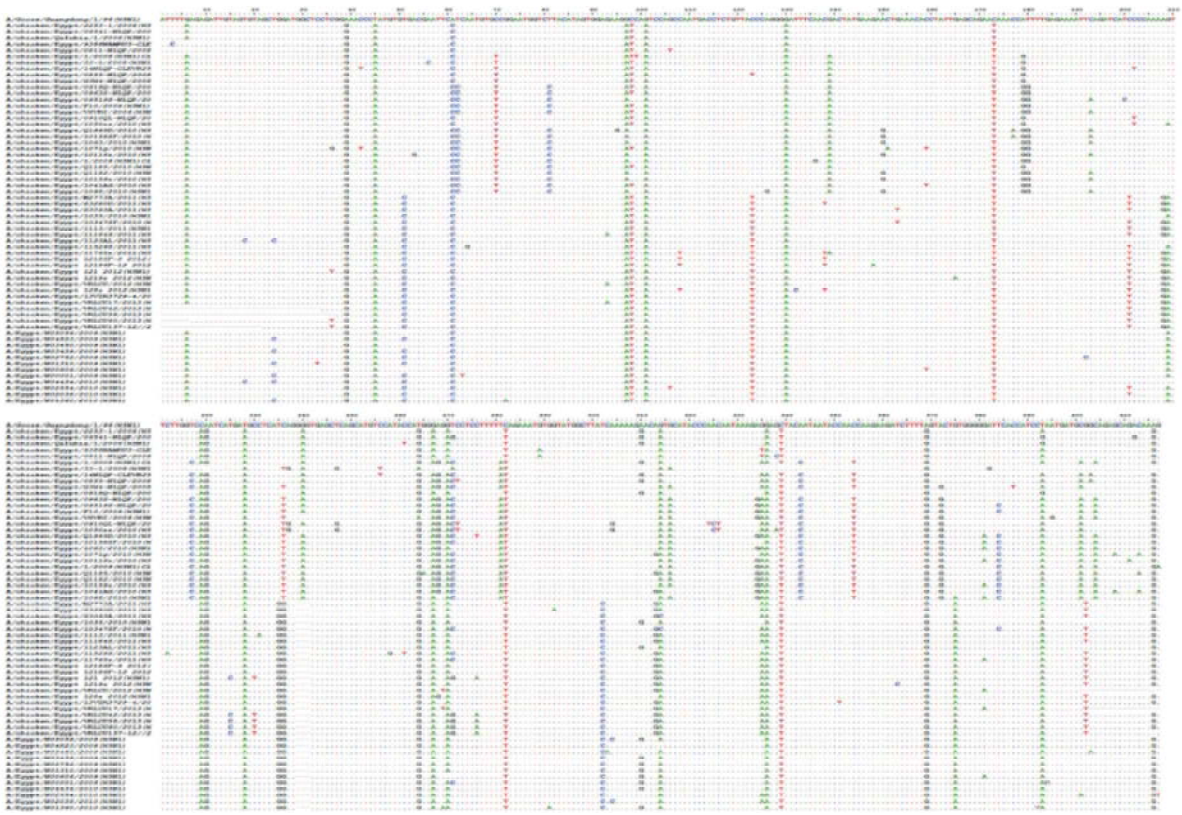

Fig. 1: Multiple nucleotide sequences alignment of the HA gene of our circulating Egyptian strains during 2013 in comparison with other representative strains of H5N1AIVs circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence

Fig. 2: 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 aminoacid sequence

Genbank Accession Number: GenBank accession numbers were received from web submission and were as follow; KF360361. 1, KF360362. 1, KC815530. 1, KF360360. 1, KF715072. 1, KJ004001. 1.

DISCUSSION

Highly pathogenic avian influenza A (H5N1) infections are continuing to affect poultry and humans in Egypt. Certainly, the risk of emergency of influenza (H5N1) pandemic could be greater in Egypt than other countries [25]. Continuous transmission of the virus among domestic poultry and on to humans in Egypt over the past five years is worrying. It is thought that H PAI viruses are generally thought to arise in poultry after infection of domestic birds by LPAI H5 and H7 viruses from wild bird reservoir [26, 27]. The evolution of influenza viruses is a continuing process that involves viral and host factors. H5N1 influenza viruses have repeatedly affect humans causing deaths of more than 50% of the humans which they have infected became of public concern [27]. However, the mechanism of interspecies transmission ofH5N1 viruses remains unclear. Although human-to-human transmissibility for H5N1influenza viruses have not detected, it is possible through reassortment and/or by mutation occur adaptation to humans. The possibility of future pandemic may be triggered by a wholly avian virus without occurrence of reassortment [10].

Epidemiological surveillance for H5N1 AIVs in Egypt revealed there was no obvious clustering observed and the viruses continued to spread across the country duringthe 2006/2007 [28]. During 2007/2008 season, variant escape mutant subclade 2.2.1.1 was emerged mainly in vaccinated farms and have extensive amino acids substitutions inor adjacent to the antigenic sites of the HA protein which enable continuous circulation of the virus among commercial poultry [13, 21] with minor

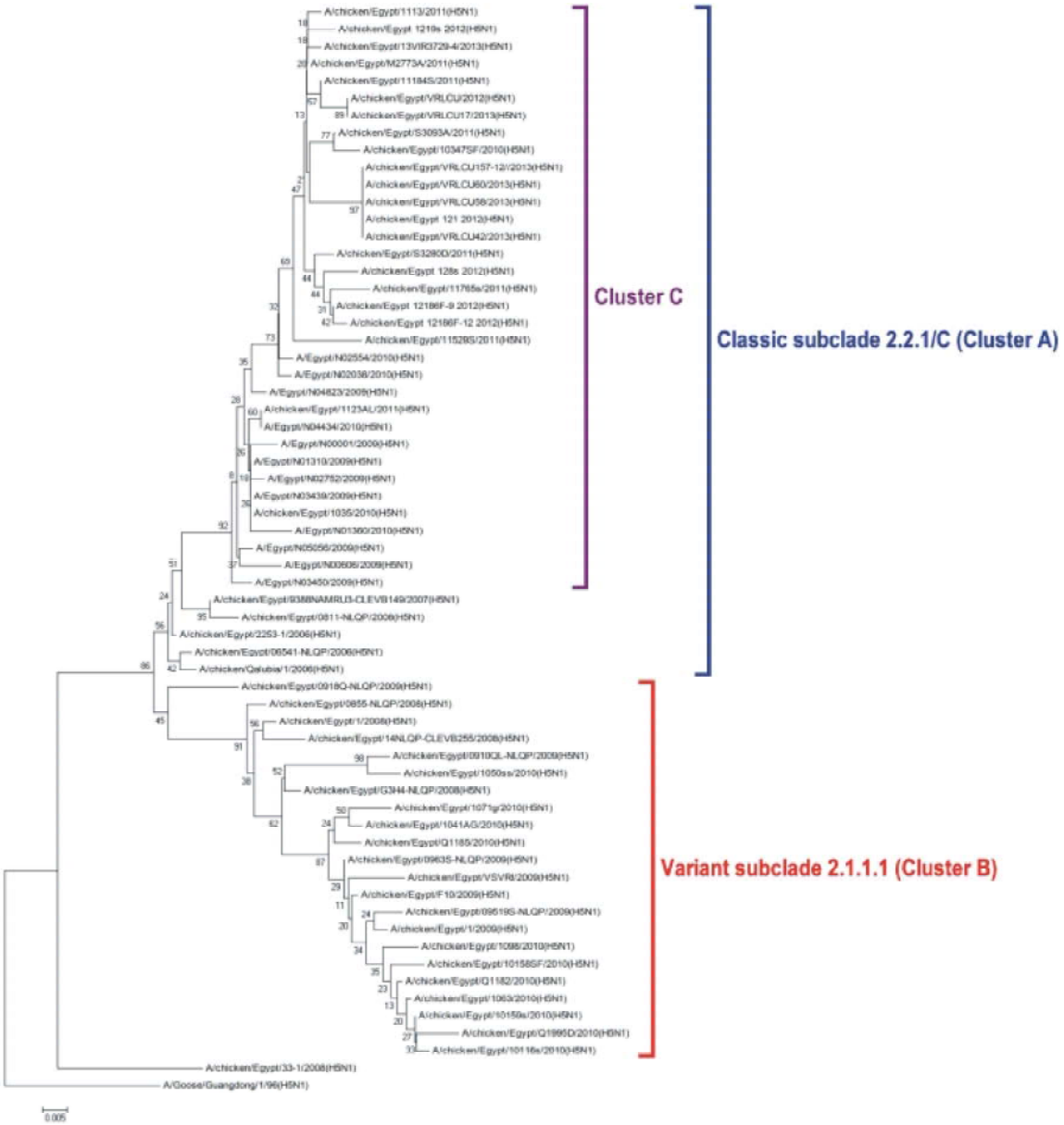

Fig. 3: Neighbor joining Phylogenetic rooted tree (A/Goose/Guangdong/1/96(H5N1))of HA gene based on nucleotide sequence showing the clustering of six Egyptian H5N1AIVs during 2013 with other representative H5N1 AIVs isolated both avian and human cases. The numbers at the nodes represent bootstrap values. Scale bar represents the number of substitutions per site. The year of isolation and geographical origin of the virus sequences are included in the tree. The tree was generated by Mega4 software program

divergence within the classicsubclade 2.2.1/C. Classic subclade 2.2.1/C viruses were found widely in backyard poultry, then continue to mutate, evolve and circulate leading to subdivision and diversities into multiple subgroups that harbor mutations in the HA and NA proteins which responsible for decreasing virulence in mammals as a step towards human adaptation [29, 30]. In 2010/2011, the classical viruses were prevailing mainly in the household and backyard birds while the variant viruses were prevailing in the commercial vaccinated farms. During the 2011/2012 season, the classic sublineage appeared to be dominant phenotypic with marked decrease in variant viruses or even disappearance.

In this study, the evolutionary dynamic for six Egyptian H5N1 AIVs in commercial poultry sectors was described during 2013 season. Currently, genotyping of H5N1 AIVs is very important for screening the emergence of new sub-lineages as well as evaluating the existing vaccination programs. Nucleotide and amino acids sequences alignment of viruses under study for HA gene were conducted in correlation withgeographically related Egyptian strains to establish the genetic spectrum, origin and evolution of H5N1 AIVs in Egypt. Nucleotide identity percentage was ranged between 92% and 99% which indicate the great diversity for the circulating Egyptian H5N1 AIVs. Multiple nucleotide alignment revealed multiple substitutions at different sites of HAgene which indicate great evolution rate for Egyptian H5N1 AIVs.

The most characteristic mutation is a deletion at position 129S of the amino acidserine which first detected in a classic group during the 2007/2008 season, then continue to evolve during 2008/2009/2010 seasons and became the dominant phenotype during1/2012 [28] and 2013 seasons which confirmed in this study. Mutation at this residue was associated with a less virulent H5N1 phenotype causing mild or asymptomatic courses of infection with increased transmission in mice [31]. Amino acids alignment revealed multiple mutations at receptor binding site (RBS). Recently, deletion of RBD 129S combined with I151T in classic subclade 2. 2. 1 Egyptian genetic group increased affinity of the Egyptian viruses to mammalian receptors and retained its avian receptor specificity [30]. However, recent viruses of human origin clustered within subclade 2. 2. 1/C only [32]. There is a scientific evidence indicating that the evolution andantigenic drift of human influenza (H1 and H3 influenza A) viruses are mainly driven by multiple mutations within major antigenic sites of HA located in the receptor binding domain (RBD) [33, 34].

In this study, we found that the E KRRKKR/G motif is characteristic for classicsubclade 2. 2. 1/C (A/chicken/Egypt/1 3V IR3729-4/201 3(H5N 1)). Interestingly, other significant substitutions such as Q192R, G222L and Q224S associated with adaptation ofthe virus from avian to mammalian receptors were not observed in any Egyptian H5 gene sequences [32, 35] including our isolates in this study. The phylogenetic tree identified that the viruses under study fall within the classic subclade 2. 2. 1/C viruses [6]. Intriguingly, virus isolated from donkeys belonged to the same subclade 2. 2. 1/C [36]. Emergence of cluster C may have resulted from suboptimal vaccination in Egypt, so in this study cluster C viruses preferentially circulated in vaccinated poultry flocks. Similar findings were reported in China, where possible vaccine-escape variants emerged 1 yearafter implementation of vaccination in poultry [37].

Continuous evolution of Egyptian H5N1 viruses and emergence of new antigenic variants may be partially explained by immune pressure from the use of vaccines. Such viruses can circumnavigate vaccine-induced immunity and are called escape mutants [38,39]. Continuous circulation among hosts may allow H5N1 virus to acquire amino acid changes enabling more bird–human transmission and eventually human to human transmission. In conclusion, circulation of HPAIV H5N1 in vaccinated birds continues the poultry industry in Egypt. Also, periodic updating for H5N1 influenza viruses classification for H5N1 viruses based on HA evolution has being required, making expansion for classification dynamic as the virus has expanded within several disparate ecosystems and along distinct evolutionary trajectories. Indeed, rapid emergence of HPAIV H5N1 lineages in Egypt demands continuous and comprehensive surveillance of viral genetic changes among commercial poultry sectors.

ACKNOWLEDGMENTS

We would like to express my deepest gratitude to all staff of the Virology Department, Faculty of Veterinary Medicine, Cairo University for their help whenever itis needed.

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