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SHORT COMMUNICATION

Re-Emergence of a Novel H5N1 Avian Influenza Virus Variant Subclade 2.2.1.1 in Egypt During 2014

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Summary

Large-scale surveillance is crucial for understanding the evolution and the emergence of avian influenza viruses (AIVs) in endemic areas. Circulation of highly pathogenic avian influenza (HPAI) subtype H5N1 is continuously causing significant economic losses to the Egyptian poultry industry and is a threat to public health. In this report, a HPAI H5N1 strain (A/chicken/Egypt/Fadllah-7/2014) was detected from a vaccinated flock showing clinical signs of infection. Genetic characterization of the isolate indicated a high level of nucleotide identity (95–98%) with variant and classical groups of H5N1. Moreover, multiple-nucleotide and amino acid alignments revealed several prominent and characteristic substitutions in the surface glycoprotein, which may have biological relevance to the pathobiology of the virus. Phylogenetic analysis demonstrated that the reported isolate closely relates to H5N1 AIVs subclade 2.2.1.1 in spite of no reports of this subclade since 2011 from AI reported cases in Egyptian avian species. In conclusion, our results highlight the re-emergence of a novel H5N1 AIV variant subclade 2.2.1.1 that could escape immunity induced by vaccines. This discovery illustrates the importance of continuous monitoring of poultry in this country for controlling AIV including identifying sources of vaccine seed viruses.

Introduction

Endemic infections or sporadic outbreaks of avian influenza viruses (AIVs) are continuously being reported in poultry populations, around the globe. Human cases of highly pathogenic avian influenza (HPAI) virus subtype H5N1, also known as 'bird flu', were first reported in Hong Kong in 1997. This raised serious concerns about emerging zoonotic infections and the possibility of H5N1 causing the next human influenza pandemic (Gao and Wang, 2006; Swayne, 2008).

In Egypt, the H5N1 was declared endemic in Egypt since 2008. The first outbreak of HPAI H5N1 in Egypt was reported in February 2006 (Aly et al., 2008). Officially, a total of 346 cases of avian influenza A (H5N1) infections in humans have been reported, including 116 related deaths (WHO, 2015). Since the first report in 2006, surveillance of HPAI viruses and an assessment of their impact on public health have been practised in Egypt by the Egyptian government through the General Organization for Veterinary Services (GOVS) in conjunction with the Central Laboratory for Quality Control of Poultry Production (Vergne et al., 2012; El-Sayed et al., 2014).

Phylogenetically, all Egyptian HPAI H5N1 viruses can be classified into two distinct groups, variant and classical groups, both belonging to clade 2.2.1., cocirculating in Egyptian poultry (Arafa et al., 2010; Cattoli et al., 2011a,b). It has been suggested that viruses of the classical group are genetically similar to the H5N1 viruses introduced in Egypt during 2006 (Aly et al., 2008). Interestingly, viruses of the

classical group are most prevalent among backyard flocks and can cause human infection (Abdel-Moneim et al., 2009, 2010). Viruses of the variant group emerged during 2007/2008 seasons, and became prevalent among vaccinated commercial poultry farms in Egypt (Arafa et al., 2012). During 2008/2009, both variant and classic groups (with subdivision into multiple subgroups) were prominent and continued to circulate until 2009/2010. It was very clear in these two seasons that the classic group was prevailing in household and backyard birds, while the variant viruses were prevailing in commercial vaccinated farms. During the 2010/2011 seasons, the classic sublineage appeared to be dominant with a marked decrease in variant viruses; only two cases from variant versus 13 classic from commercial chickens (Arafa et al., 2012).

As influenza viruses are known to adapt to new hosts owing to high and unpredictable evolution levels (Spackman et al., 2007), it is possible that the variant strains may have been a vaccine-escape mutant of the original 2006 virus (Abdel-Moneim et al., 2009; Arafa et al., 2010).

Due to rapid evolution of influenza viruses, continuous isolation and molecular characterization of circulating strains needs to be performed, particularly in places such as Egypt where HPAI H5N1 and other AIVs are prevalent, poultry outbreaks and human infections occur frequently. Avian influenza was suspected to have caused an outbreak in a vaccinated commercial poultry flock in Egypt in November 2014. This study was conducted to ascertain the molecular and genetic features of the viruses isolated in this outbreak.

Materials and Methods

Sampling history

Samples were collected on 25 November 2014 from a commercial layer farm located in the El-Menoufia, Egypt (Nile Delta). The flock consisted of 22 000 Hy-Line white layers. Birds were kept in cages, and were 6 weeks at the time of the outbreak. The flock has been vaccinated in their first day at hatchery with a commercially available recombinant herpes virus of turkey (HVT) vaccine for both H5N1 AI (Vectormune® AI; Ceva) and Newcastle disease virus (NDV) (Vectormune® NDV; Ceva). The owner conducted vaccination of birds as part of a routine vaccination practices in the area while it is contraindicated for concomitant vaccination of HVT vaccines according to manufacturers' instructions (http://www.vectormune.com/Vectormune-R-ND/Vectormune-R-ND-product-information and http://www.vectormune.com/Vectormune-R-AI/Downloadable-Vectormune-R-AI-product-brochure). At 7 weeks of age after the appearance of clinical signs (i.e. drop in egg production, severe respiratory manifestations with high morbidity rate), ten tracheal and cloacal swabs were collected randomly from sick and apparently healthy birds. All swabs from the cloaca and trachea were pooled into one sample for each sample type. Pooling was performed according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2009) for subsequent analysis. Ten serum samples were also collected for serological investigations.

Virus isolation and propagation

Pooled samples from the cloaca and trachea were pooled into 2 ml of phosphate buffer solution (PBS) containing antibiotics, and then filtered through 0.45-μm syringe filter. Two pooled samples were inoculated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) for virus isolation. The inoculums (0.2 ml) were administered into the allantoic cavity of each ECE. Inoculated eggs were incubated at 37°C for 5 days with daily candling to monitor any mortality.

Haemagglutination (HA) and haemagglutination inhibition (HI) assays

Harvested allantoic fluid was collected in sterile tubes. Slide HA tests were performed for rapid detection of HA activity, and followed by standard quantitative HA tests. To further characterize the virus, the HI assays were carried out using OIE reference H5N1 antiserum (OIE, 2009).

Viral RNA extraction and RT-PCR assay

RNA was extracted from positive pools (n = 2), using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. One-step RT-PCR was performed using One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions (Rohaim et al., 2014). The amplified products (5 μl) were run on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide for nucleic acid visualization.

Sequencing and phylogenetic analysis

The amplified HA gene products were purified using a PCR Purification Kit (Qiagen, Valencia, CA, USA) and were sequenced using gene-specific primers (primer sequences are available upon request). Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM® 3100 Sequencer. Comparative analysis of HA gene sequences of the H5N1 viruses was carried out and compared with available sequences in the National Center for Biotechnology Information (NCBI) influenza virus resources database. Phylogenetic analysis for full-length HA gene was performed using MEGA5 software (Tamura et al.,

2007). The evolutionary history was inferred using the neighbour-joining method and the reliability of each tree branch was estimated by performing 1000 bootstrap replicates. The obtained sequence for H5N1 was submitted to the GenBank and is available under the accession number KP326324.

Challenge experiment

Day-old SPF chicks were randomly separated into three groups of ten birds each: one treated, one control positive and one control negative. The treated group was vaccinated with Vectormune® AI at day one of age as recommended by the manufacturer's instructions, and challenged at 28 days of age with our field isolate under study [A/chicken/Egypt/Fadllah-7/2014(H5N1)], diluted in cell culture medium containing 10% foetal calf serum (FCS) by the oculo-oronasal route. The effectiveness of vaccination was evaluated on the basis of clinical protection (morbidity and mortality) and measurement of challenge virus shedding by real-time RT-PCR (rRT-PCR) on cloacal swabs from the challenged chicks (Abdelwhab et al., 2010).

Immune response to vaccination was monitored by hemagglutination inhibition test, and serum samples were collected at hatch and at 28 days of age (before the challenge). In addition, the intravenous pathogenicity index (IVPI) was determined in SPF chickens according to standard protocols (OIE, 2009). Challenge experiment was conducted in BSL3 chicken isolators at The Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Egypt.

Results and discussion

Rapid divergence and higher evolution rates for avian influenza viruses (e.g. H5N1) have accelerated the importance of surveillance and the need for the epidemiological data, which are fundamental in devising effective control strategies. Epidemiological AIV surveillance may be carried out based on serological and/or molecular detection. However, serological data may not be useful during active poultry outbreaks and epidemic situations. In the present study, molecular characterization of H5N1 AIV was performed on two pooled samples based on RT-PCR for full-length HA gene, followed by sequencing and phylogenetic analysis. During daily observation for the inoculated (ECE), it was observed that all embryos died within 24–48 h post-inoculation associated with diffuse haemorrhagic lesions. The allantoic fluid was harvested and tested for HA activity according to the OIE recommendations (OIE, 2009) and the titres ranged between 6 and 8 $\log^2$. HI assays were conducted on the serum samples using two different antigens and a titre of 6.42 $\log^2$ with variant 2.2.1.1 strain [A/chicken/13VIR952/2011(H5N1)] and 3.42 $\log^2$ with the classical 2.2.1/C strain [A/chicken/13VIR3729-4/2013(H5N1)] was observed. HI assays were conducted on the serum samples using the isolate under study [A/chicken/Egypt/Fadllah-7/2014(H5N1)] and a titre of 7.4 $\log^2$ was detected that confirmed the cross-reactivity between the virus and the serum. The sequences obtained for two pooled samples were identical when subjected to basic local alignment tool (BLAST) in the GenBank (NCBI) to identify percentage similarity and relationship with the H5N1 AIVs. Multiple-nucleotide alignment for the HA gene revealed the highest similarity percentage (98%) with the variant subclade 2.2.1.1. The polybasic cleavage site is thought to be the primary virulence determinant for HPAIs (Neumann and Kawaoka, 2006; Garten and Klenk, 2008; Abdelwhab and Hafez, 2011; Kuchipudi and Chang, 2015). Mutation trend analysis for the HA gene revealed numerous mutations at critical sites such as the binding site, antigenic sites, and cleavage site (Table 1). Amino acid changes that are associated with HI reactivity of H5 viruses were recorded in the HA protein of the studied isolate (Table 1). Characteristic motifs were identified in the HA glycoprotein of the reported isolate, which include a stretch of sequence (EGRRKKRG), present in HA cleavage site of variant subclade 2.2.1.1 with a single amino acid (G) substitution

Table 1. Amino acid variations at critical sites of HA proteins of the AIV[a]

Amino acid mutations of the antigenic sites					Cleavage site (CS)	Receptor binding site (RBS)	Substitutions in the HI activity sites
Site A	Site B	Site C	Site D	Site E			
119 (K), 120 (N), 138 (Q), 140 (K), 141 (P), 161 (K), 162 (E)	124 (D), 129 (L), 151 (I), 155 (T), 156 (T), 184 (D), 188 (T), 189 (R)	282 (V)	94 (N), 226 (V)	83 (I), 84 (N)	323 (G), 324 (E), 325 (G), 326 (R), 327 (R), 328 (K), 329 (K), 330 (R)	189 (R), 217 (P)	84 (N), 94 (N), 120 (N), 124 (D), 126 (E), 129 (L), 138 (Q), 140 (K), 155 (T), 156 (T), 162 (E), 184 (D), 185 (A), 189 (R), 226 (V), 263 (T), 310 (R), 446 (K)

[a]H5 numbering is according to Wibawa et al. (2011). The mutations are within the reported isolate [A/chicken/Egypt/Fadllah-7/2014(H5N1)].

different from (EKRRKKRG) of subclade 2.2.1/C H5N1 (Stech et al., 2009; Bogs et al., 2010; Munster et al., 2010). Secondly, a substitution of serine at 129 (S129) amino acid position in the HA glycoprotein was initially detected in a classic group during the 2007/2008 outbreaks in Egypt. The isolate in the current study (variant subclade 2.2.1.1) has shown a substitution of S129L (Table 1) that is may be essential benchmarks of pathogenicity and thus require future biological and reverse genetics studies to investigate their potential roles in the evolution and biology of Egyptian H5N1 AIVs. It is well known that the affinity of H5N1 virus to avian type α2-3 and mammalian-like α2-6 linked sialic acid receptors seems to be governed by a number of residues in the HA protein including serine at position 129 which is a part of the receptor binding domain (RBD) (Duvvuri et al., 2009). It has been found that the Egyptian H5N1 viruses had a potential to use mammalian receptors resembling seasonal H1N1 virus (Veljkovic et al., 2009). Substitution at residue 129 was associated with a less virulent H5N1 phenotype causing milder or asymptomatic courses of infection and increased transmissibility in mice (Kaverin et al., 2007). Interestingly, other significant substitutions such as Q192R, G222L and Q224S are associated with adjustment of the virus from avian to mammalian receptors (Matrosovich et al., 1999). Unfortunately, there is a paucity of information on subclinical spread among people in Egypt, particularly among those that are in close contact with infected backyard birds. However, since late 2008, symptomless cases infected with mild virulent H5N1 in Egypt have raised concerns that the virus might be adapted to Egyptians without getting sick (Anon, 2009). Two major antigenically and genetically distinct groups of H5N1 viruses (classic group of subclade 2.2.1/C, and variant group of 2.2.1.1) have been cocirculating in the Egyptian vaccinated commercial poultry sectors since 2007 (Carrat and Flahault, 2007; Abdelwhab and Hafez, 2011). Based on the phylogenetic analysis, conducted to better understand the genetic relation and evolution of Egyptian H5N1 viruses, we showed the isolate under study was clustered in the clade 2.2.1 of HPAI H5N1 viruses (Fig. 1). High-resolution genetic and evolutionary analysis revealed the re-emergence of variant escape mutant 2.2.1.1 during 2014. Interestingly, despite continuous reports of HPAIs in Egypt, this subclade has not been reported in the Egyptian poultry since 2011. Interestingly, despite continuous reports of HPAIVs in Egypt, this subclade has not been reported in the Egyptian poultry since 2011 in spite of high prevalence of subclade 2.2.1/C due to inadequate surveillance (Kayali et al., 2014). Our results indicate that variant escape mutant 2.2.1.1 may have progressively accumulated novel mutations over the last 3 years in vaccinated flocks and these substitutions could facilitate the adaptation of the virus to switch host from avian to mammalian in the future especially in case of mixed infections with variant and classical subclades H5N1 AIVs.

The Egyptian virus [A/chicken/Egypt/Fadllah-7/2014 (H5N1)] had an IVPI of 2.9, which is characteristic for the highly pathogenic viruses inducing 100% mortality within 3 days in non-vaccinated control chickens by the oculo-oronasal route. In this study, when specific pathogen-free (SPF) chicks vaccinated with Vectormune® AI vaccine evoked high HI titre (7.0 ± 0.8 log 2) and were protected against challenge with an Egyptian H5N1 virus belonged to the variant (2.2.1.1) viruses. To evaluate the protective efficacy of Vectormune® AI vaccine-induced immunity, challenge experiments using isolate under study [A/chicken/Egypt/Fadllah-7/2014(H5N1)] were carried out according to the standard protocols. Challenged chicks were clinically protected regardless of the challenge virus with the ability to reduce viral excretion below detection limit after challenge while control groups were 100% mortality for control positive and 0% mortality for control negative. Therefore, we assume that suppression of the immune system (i.e. ingestion of mycotoxins) (Hegazy et al., 2011) or mixed infections with other viruses and or bacteria (Haghighat-Jahromi et al., 2008; Hussein et al., 2014) could be responsible for such weak immune response and subsequently lack of protection as well as concomitant vaccination with Vectormune® AI and Vectormune® NDV is contraindicated according to the manufactures' instruction.

During the last 5 years, H5N1 viruses in Egypt have continued to evolve. Mass vaccination campaigns have failed to control HPAI H5N1 outbreaks in Egypt (Hafez et al., 2010). There is an urgent need to study the relationship between genetic evolution and selection of influenza virus phenotypes. During 2010/2011 seasons, epidemiological surveillance data revealed that the classic viruses were prevailing in household and backyard birds while the variant viruses were prevailing in commercial vaccinated farms (Arafa et al., 2012). Continuous evolution of H5N1 viruses and the emergence of new variant strains may be partially explained by immune pressure caused by vaccination (Abdel-Moneim et al., 2011; Grund et al., 2011). Such variants can circumnavigate vaccine-induced immunity and are thus escape mutants (Cattoli et al., 2011b). Rapid evolution rate and occurrence of antigenic drifts at the antigenic sites of HA gene may favour the emergence of new sublineages and/or potential pandemics in the country.

In conclusion, despite mass vaccination using different commercial vaccines (inactivated and recombinant), the Egyptian poultry flocks still exhibit observable distress and mortality that may reach up to 100%. This suggests that the updated poultry vaccine may be necessary with re-evaluation of routine vaccination practices in Egypt and the effi-

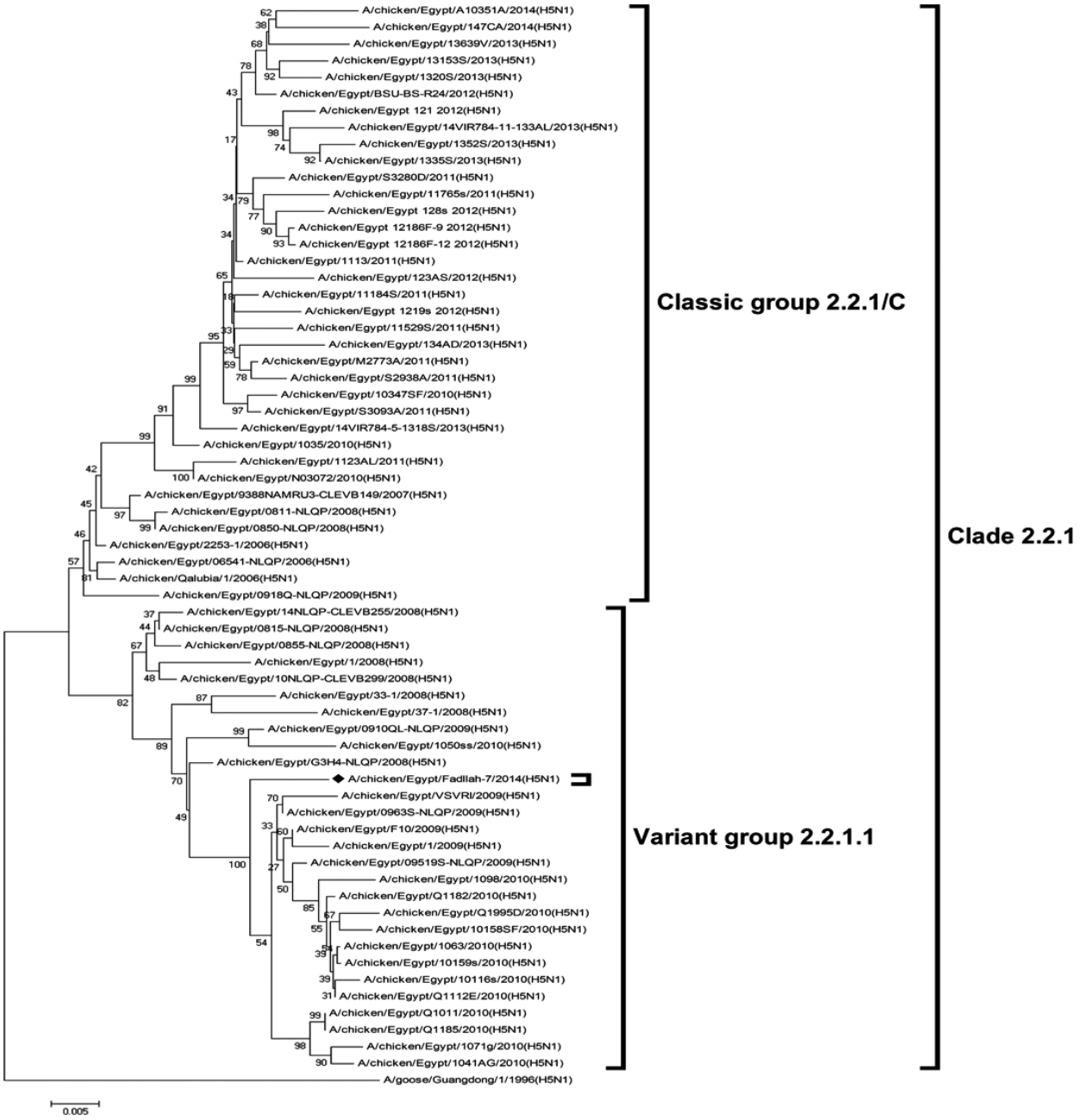

Fig. 1. Phylogenetic analysis of HA gene of H5N1 AI Egyptian isolate A/chicken/Egypt/Fadllah-7/2014(H5N1) together with H5N1 sequences available in public domains and Egyptian representative H5N1 strains. The tree was constructed using MEGA5 software, using bootstrap analysis (n = 1000) with A/goose/Guangdong/1/1996(H5N1) as an out-group. All Egyptian viruses belonged to the avian influenza clade 2.2.1. The reported isolate is marked with black filled square and bootstrap values are shown above each branch.

cacy of vaccine to evoke protective immune response in the field still needs to be investigated. Therefore, large-scale surveillance of AIVs, especially zoonotic H5N1 in endemic areas, should be expanded to better understand the evolution of influenza viruses both in wild birds and in commercial poultry. Due to endemic status of H5N1 influenza viruses in poultry and limitations of the reporting system in Egypt, it is difficult to assess whether the fluctuating epidemiological patterns of the emerging virus are due to altered biological properties in poultry or due to increased incidence of infections especially with the recent reporting of mixed infection with other respiratory viruses. It is therefore essential to continuously monitor the epidemiology, pathogenicity and transmissibility of these viruses and

to evaluate the routine vaccination practices to ensure the manufacturers' instructions are being followed consistently. Concerted efforts of both veterinary and public health authorities are essential to interrupt virus circulation in Egyptian poultry and to minimize the risk to public health.

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