Infection, Genetics and Evolution 58 (2018) 56–65

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Multiple introductions of reassorted highly pathogenic avian influenza viruses (H5N8) clade 2.3.4.4b causing outbreaks in wild birds and poultry in Egypt

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ARTICLE INFO

Keywords:
Avian influenza
Subtype H5N8
Clade 2.3.4.4
Reassorted virus
Wild birds
Domestic poultry
Egypt

ABSTRACT

Recently, an increased incidence of outbreaks of highly pathogenic avian influenza (*HPAI*) H5N8 in poultry linked to infected migratory birds has been reported from different European, Asian and African countries.

In Egypt, incursion of *HPAI* H5N8 virus of clade 2.3.4.4b has been recently registered. Full genomic characterization of 3 virus isolates from wild birds and poultry (backyard and commercial farm sectors) showed high nucleotide similarity among the HA, NA, M, and NS gene segments of the three Egyptian *HPAI* H5N8 viruses, indicating that they are descendants of a common ancestral virus. However, the analyzed Egyptian H5N8 viruses revealed distinct genotypes involving different origins of the PB2, PB1, PA and/or NP segments. In genotype-1 represented by strain A/common-coot/Egypt/CA285/2016 the PB2 and NP segments showed closest relationship to H5N6 and H6N2 viruses, recently detected in Italy. The second is replacement of PB1 and NP genes A novel reassortant, represented by strain A/duck/Egypt/SS19/2017, showed an exchange of PB1 and NP genes which might have originated from H6N8 or H1N1 and H6N2 viruses. Finally, replacement of PA and NP genes characterized strain A/duck/Egypt/F446/2017. Bayesian phylogeographic analyses revealed that Egyptian H5N8 viruses are highly likely derived from Russian 2016 *HPAI* H5N8 virus (A/great_crested_grebe/Uvs-Nuur_Lake/341/2016 (H5N8)) and the reassortment likely occurred before incursion to Egypt.

1. Introduction

Highly Pathogenic Avian Influenza (*HPAI*) is a highly contagious avian disease, may cause serious economic losses in poultry industry and poses a potential threat to public health (Neumann, 2015). Since the emergence of *HPAI* virus H5N1 A/goose/Guangdong/1/1996 (GS/GD96) in China in 1996, descendants of this strain continue to spread among avian species and their hemagglutinin (HA) has evolved into multiple distinct phylogenetic clades, subclades and lineages in different geographic locations mainly in Southeast Asia (Smith et al., 2015). The evolution of these viruses included numerous reassortment events with other avian influenza viruses (AIV) leading to the emergence of a multitude of genotypes and several subtypes (Dalby, 2016).

The ancestral strain of HPAIV of subtype H5N8 originated from breeding ducks in China in 2010 which founded clade 2.3.4.4 of GS/GS96 viruses (Smith et al., 2015). Further in January 2014, new reassortant strains of this lineage were reported in both wild birds and domestic poultry in South Korea (Lee et al., 2014), which subsequently spread to other countries in Asia, Europe, and North America by the end of 2014 (Lee et al., 2015; Saito et al., 2015). Most recently, in 2016/2017, yet another novel reassortant virus of clade 2.3.4.4 of subtype H5N8 was reported in wild birds at the Russian-Mongolian border in summer 2016. This virus appeared in autumn 2016 in many countries in Europe, Asia, and Africa (Dong-Hun et al., 2017; ECDC, 2016; Selim et al., 2017). Dissemination of *HPAI* H5N8 viruses to Europe, the Middle East and Africa has been linked to flyways of migratory wild birds (Dong-Hun et al., 2017; ECDC, 2016; Selim et al., 2017). Outbreaks in poultry in several European countries resulted in the culling of tens of thousands of domestic poultry within a short period of time (Fusaro et al., 2017; Pohlmann et al., 2017). Recent viruses of clade

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https://doi.org/10.1016/j.meegid.2017.12.011
Received 4 August 2017; Received in revised form 10 December 2017; Accepted 13 December 2017
Available online 14 December 2017
1567-1348/

2.3.4.4 are highly pathogenic in domestic poultry and have also shown enhanced virulence for anseriform wild birds (Pohlmann et al., 2017), but are considered to have a low risk of zoonotic transmission (Kim et al., 2014). To date, there are no reports of human cases of infection with these viruses.

Originally, *HPAI* H5N8 viruses of clade 2.3.4.4 clustered into two groups: group A (Buan-like: A/Broiler-duck/Korea/Buan2/2014) and group B (A/breeder-duck/Korea/Gochang1/2014, Gochang-like) (Lee et al., 2017). In the recent 2016/2017 wave of spread of H5N8 HPAIV, different genotypes have been detected as a result of reassortment, for examples: (i) In Russia: reassortant H5N8 had retained three gene segments (HA, NA, and NS) of Korean ancestral viruses of clade 2.3.4.4 group B while the remaining five segments originated from different avian influenza (AI) virus subtypes from Mongolia and China (Lee et al., 2017) (ii) In Germany, a novel reassortant H5N8 was reported with a similar genotype of the parental virus found earlier in Russia, however PA and NP gene segments were derived from other AIV (Pohlmann et al., 2017); the same reassortant was later found in India (Nagarajan et al., 2017) (iii) In Italy, an additional two HPAIV H5N8 reassortants evolved (either with reassorted PB2, PA, and NP or with reassorted PB2 and NP) (Fusaro et al., 2017). An incursion of *HPAI* H5N8 viruses had also been registered in Egypt in November and December 2016 when aquatic wild birds (common coot and green-winged teal) were found to be affected (Kandeil et al., 2017; Selim et al., 2017). Based on sequences of the HA and NA segment, this virus was shown to be closely related to the European *HPAI* H5N8 viruses of clade 2.3.4.4b. However, no information about the full genome of this virus has been yet described.

In the present study, full genome sequence analysis of three H5N8 viruses from a wild bird (common coot), backyard (duck), and farm poultry (duck) were carried out for better understanding the origin and the genetic characteristics of the newly emerged *HPAI* H5N8 viruses in Egypt and their genetic and phylogenetic relationship with the ongoing *HPAI* H5N8 viruses in Asia and Europe. In addition, phylogeographic analyses were included to elucidate possible spreading and introduction pathways.

2. Material and methods

2.1. Samples

Pooled oropharyngeal swabs were collected from 465 house-hold bird populations including chickens, ducks and geese from 9 governorates all over Egypt (Tables 1 & S1). Samples were obtained during a targeted surveillance on diseased poultry and were performed by the Egyptian veterinary authority from January to April 2017. All the diseased backyard duck flocks suffered from mixed clinical illness ranged from loss of appetite, nasal and ocular discharge up to nervous signs ended with death. While the chicken backyard flocks suffered from respiratory distress, nasal and ocular discharge, cyanosis in legs and face and deaths. Additional 14 pooled samples were collected from commercial chicken and duck farms from 3 governorates from cases with respiratory illness and high mortalities reached up to 70%. (Tables 1 & S1). The collected samples were submitted to the National Laboratory of veterinary quality control on poultry production (NLQP) for AIV virus detection, identification and isolation as well as virus genotyping. Additionally, the H5N8 virus A/common-coot/Egypt/CA285/2016, that was previously isolated from the first reported case of H5N8 introduced into Egypt in a common coot in late 2016 (Selim et al., 2017), was retrieved from the virus bank at the NLQP to complete its whole genome analysis for comparison.

2.2. RNA extraction and molecular diagnosis

Viral RNA was extracted from the samples using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were tested using one step RT-qPCR (Qiagen, Hilden, Germany) for the M gene of influenza type A viruses (Spackman et al., 2003) using the real-time PCR Mx3000P QPCR System (Agilent, California, USA). Positive AIV RNA was subtyped for HA and NA using specific subtyping RT-qPCR (Hoffmann et al., 2016; Monne et al., 2008).

Table 1
Geographical spread of H5N8 in Egyptian Governorates.

No	Governorate	Positive cases/total examined	
		Farm	Household
1	Sharkia	0/5	2/118
2	Dakahlia	0	1/42
3	Domiatt	0	1/19
4	Ismaelia	0/5	3/12
5	Kafr El-sheikh	0	1/45
6	Gharbia	0	1/56
7	Suiz	0	1/4
8	Cairo	1/4	0
9	Minia	0	5/122
10	Qena	0	3/47
	Total	1/14	18/465

2.3. Virus isolation

PCR-positive samples were processed for virus isolation. Virus isolation was performed by inoculation into the allantoic cavity of 10-day old specific pathogen free (SPF) embryonated chicken eggs (ECG) according to the OIE diagnostic (OIE, 2014). Collected allantoic fluid was tested by hemaglutination assay and specific RT-qPCRs (Hoffmann et al., 2016; Monne et al., 2008). Positive allantoic fluids were stored at − 80 °C.

2.4. Gene sequencing

The complete genome sequences of the two selected viruses (EG-duck/SS19, EG-duck/F446) in addition to six internal genes of the common coot virus isolate (EG-common-coot/CA285) were amplified by RT-PCR using the SuperScript III Platinum One-Step RT-qPCR (Invitrogen, California, USA) with primers described previously (Hoper et al., 2009; Naguib et al., 2015). The gene-specific RT-PCR amplicons were size-separated by agarose gel electrophoresis, excised and purified from gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Further, purified PCR products were used directly for cycle sequencing reactions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). Reaction products were purified using Centrisep spin columns (Thermo Fisher, California, USA) and sequenced on an ABI 3500 Genetic Analyzer (Life Technologies, California, USA). Sequences generated in this study were submitted to the Global Initiative on Sharing All Influenza Data (GISAID) platform under accession numbers: (i) A/Common-coot/Egypt/CA285/2016 (EPI1018188-90, EPI1018194-95, and EPI1018198) (ii) A/duck/Egypt/F446/2017 (EPI1018204-11), and (iii) A/duck/Egypt/SS19/2017 (EPI1018177-84).

2.5. Genetic and phylogenetic characterization

The obtained sequences were assembled and edited using the Geneious® software, version 9.0.5 (Kearse et al., 2012). A BLAST search was performed using GISAID platform, and sequences used in this study have been retrieved from the GISAID database for representative H5N8 and other similar viruses. Alignment and identity matrix analyses were done using MAFFT (Katoh and Standley, 2013). Phylogenetic analyses were based on maximum likelihood methodology based on Bayesian (BIC) criterion after selection of the best fit models using IQ-tree software version 1.1.3 (Nguyen et al., 2015). Trees were finally viewed and