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Pathogenicity of the Egyptian A/H5N1 avian influenza viruses in chickens

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ABSTRACT

Long-term circulation of highly pathogenic avian influenza H5N1 viruses of clade 2.2.1 in Egyptian poultry since February 2006 resulted in the evolution of two distinct clades: 2.2.1.1 represents antigenic-drift variants isolated from vaccinated poultry and 2.2.1.2 that caused the newest upsurge in birds and humans in 2014/2015. In the present study, nine isolates were collected from chickens, ducks and turkeys representing the commercial and backyard sectors during the period 2009–2015. The subtyping was confirmed by hemagglutination inhibition (HI) test, RT-qPCR and sequence analysis. The Mean Death Time (MDT) and Intravenous Pathogenicity Index (IVPI) for all isolates were determined. Sequence analysis of the HA gene sequences of these viruses revealed that two viruses belonged to clade 2.2.1.1 and the rest were clade 2.2.1.2. Antigenic characterisation of the viruses supported the results of the phylogenetic analysis. The MDT of the isolates ranged from 18 to 72 h and the IVPI values ranged from 2.3 to 2.9; viruses of the 2.2.1.1 clade were less virulent than those of the 2.2.1.2 clade. In addition, clade-specific polymorphism in the HA cleavage site was observed. These findings indicate the high and variable pathogenicity of H5N1 viruses of different clades and host-origin in Egypt. The upsurge of outbreaks in poultry in 2014/2015 was probably not due to a shift in virulence from earlier viruses.

1. Introduction

Since 1996/1997, highly pathogenic avian influenza virus (HPAIV) of H5N1 subtype (A/H5N1) diversified into 10 phylogenetic clades (designated clade 0 to 9) based on the sequence homology of the hemagglutinin (HA) gene. Viruses in clade 2.2 were widespread in poultry in many countries in Asia, Europe and Africa [1,2]. Nigeria was the first African country to report an HPAIV H5N1 of clade 2.2.1 in domestic poultry in 2006. Since then, HPAIV were isolated from poultry in eleven African countries including Sudan, Djibouti, Niger, Burkina Faso, Cote d'Ivoire, Ghana, Togo, Benin and Cameroon [3]. A/H5N1 of clade 2.2.1 was detected from domestic birds in Egypt in February 2006 [4] and continues to cause significant losses in poultry industry [5]. Vaccination of poultry using numerous diversified H5 vaccines was used extensively in the commercial sector, resulting in diversification of the endemic virus into two major clades: 2.2.1.1 and 2.2.1.2 based on the phylogenetic analysis of the HA gene sequences. The latter clade was isolated from human, non-vaccinated backyard birds and commercial poultry, whereas viruses in clade 2.2.1.1 exhibiting significant antigenic drift from the vaccine strains were isolated from vaccinated commercial birds [6]. Pathogenicity of A/H5N1 was contributed to by many virus genes, however the cleavability of the HA protein to HA1 and HA2 subunits and the distribution of HA-activating proteases within the host were recognized as the major virulence factors [7]. Also, other gene segments (e.g. PB2, PA, NP, and/or NS1) may modulate the virulence of A/H5N1 in birds and mammals [8–12]. Histopathological lesions of A/H5N1-infected birds involve inflammation and/or necrosis of different organs, mainly lung, trachea, brain, pancreas and cecal tonsils [13].

Abbreviation: ECE, Embryonated chicken egg; IVPI, Intravenous pathogenicity index; MDT, Mean death time; HPAIV, Highly pathogenic avian influenza virus.

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The pathogenicity of the currently circulating Egyptian H5N1 viruses (clade 2.2.1.2) is poorly understood. In October 2014 to March 2015, Egypt experienced an upsurge of A/H5N1 infections in poultry and subsequently humans. The incidence of A/H5N1 increased dramatically and over 400 outbreaks were reported in the commercial and household poultry [5]. Whether the virus acquired a higher virulence than previously circulating A/H5N1 viruses is not yet known; given the fact that the recent isolates showed no significant antigenic drift compared to isolates from previous years [5]. Therefore, reasons other than vaccine-mismatch could be involved. In the present study, the pathogenicity of the Egyptian A/H5N1 viruses from different clades, host origin and production sectors from 2009 to 2015 was determined using the standard intravenous pathogenicity index (IVPI) in chickens.

2. Materials and methods

2.1. Viruses

Nine viruses were obtained from the repository of the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Egypt as shown in Table 1. They were isolated from swabs collected from chickens (n = 7), turkeys (n = 1) and ducks (n = 1) in the backyard sector (n = 5) and commercial enterprises (n = 4) during the period 2009–2015 (Table 1). Samples were collected during a national surveillance according to the ministerial degree 221/2006 issued by the Minister of Agriculture, Egypt. Two viruses A63/10 and AS3/12 were isolated from commercial flocks that were vaccinated with H5N1 vaccines. The vaccination history of AD6/11 and FL4/15 viruses was not available. Backyard birds were not vaccinated.

2.2. Virus propagation and titration

Specific pathogen free (SPF) embryonated chicken eggs (ECE) were purchased from the Egyptian S.P.F. Eggs Production Farm (Nile SPF), El-Fayoum Governorate, Egypt. Eggs were incubated at 37 °C for 9–11 days and viruses were inoculated via the allantoic sac according to the OIE guidelines [14]. Inoculated eggs were candled daily for 3–5 successive days. Allantoic fluids were collected from eggs and HA titres were estimated using V-bottom microwell plates against 1% chicken erythrocytes obtained from SPF chickens as previously described [14]. Allantoic fluids were platted on blood agar. Bacteria free allantoic fluid was aliquoted and stored at −80 °C until used. Mean death time (MDT) of the viruses was determined by inoculation of allantoic cavity of 9–11 ECE and observation of mortality every 18 h. The viral infectivity of each strain was determined by serial titration in 10–11-day-old embryonated eggs, and was expressed as 50% of the egg infective dose (EID50)/mL using the method reported by Reed and Muench [15]. Antigenic characterisation of A/H5N1 in this study was done using the hemagglutination inhibition (HI) test according to the OIE protocol [14]. The antisera were prepared in the RLQP animal facilities. Egyptian H5N1 viruses from different clades were inactivated by 0.01% formalin (Merck, Germany). Montanide® ISA 720 (Seppic Inc., France) as adjuvant was added to the antigens according the manufacturer recommendations. Three-week-old SPF chickens were injected intramuscular and were euthanized three weeks later.

2.3. Sequence and phylogenetic analyses

Using One-Step RT-PCR Kit and generic primers [16], the full-length HA genes were successfully amplified from all viruses. Amplicons were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequences were obtained using an ABI Big Dye Terminator v.3.1 Sequencing Kit (Perkin-Elmer, Foster city, CA) in Applied Biosystems 3130 genetic Analyzer (ABI, USA). The resulting sequences were submitted to a BLAST search to confirm the high similarity with other H5N1 viruses in GenBank [17]. Sequences generated from present study are currently available on GenBank and their accession numbers are provided in Table 1. Phylogenetic relatedness of sequences generated in this study to other A/H5N1viruses from Egypt was assessed by retrieving relevant gene sequences from GenBank. Nucleotides and deduced amino acids (aa) were analysed using MAFFT [18] and further edited by BioEdit 7.1.7 [19]. Maximum likelihood trees were constructed after selection of the best fit model and MrBayes as implemented in Topali v.2 software [20]. The phylogenetic tree was further edited by Inkscape 2.0 (Free Software Foundation, Inc., Boston, USA).

2.4. Intravenous pathogenicity index (IVPI)

Assessment of pathogenicity of the selected viruses was estimated by IVPI by inoculation of 0.2 ml of bacterial free allantoic fluid intravenously of ten 6-week-old SPF chickens [14]. Ten chickens per group were housed in biosafety level 3 isolators with HEPA filters and ventilated under negative pressure. The handling of birds was conducted in compliance with the principles and specific guidelines presented in the Guidelines for the Care and Use of Agricultural Animals in Research and Teaching [21]. The allantoic fluid was thawed and the HA titres were estimated immediately before virus inoculation in SPF chickens. Each chicken received 0.2 ml of the diluted virus (1:10) slowly through the brachial vein in the direction of blood flow. The birds were examined at 24-h intervals for 10 days for respiratory disorders, depression, diarrhoea, cyanosis of comb or wattles, facial and/or head oedema, nervous

Table 1
Pathogenicity of different A/H5N1 viruses isolated from poultry in Egypt.

No.	Virus	Abbreviation	Clade	Production sector	Accession no	EID50 log10	MDT on ECE*	Pathogenicity Index**	Virus excretion***
1	A/Chicken/Egypt/0918-NLQP/2009	A18/09	2.2.1.2	Backyard	JF746741	7	48hr	2.69	4.4
2	A/Chicken/Egypt/1063/2010	A63/10	2.2.1.1	Farm	HQ198269	7	72hr	2.39	3.5
3	A/Chicken/Egypt/1112/2011	A12/11	2.2.1.2	Backyard	JN807812	6	48hr	2.60	4.2
4	A/Chicken/Egypt/116AD/2011	AD6/11	2.2.1.1	Farm	JN807843	6	72hr	2.33	4.5
5	A/Chicken/Egypt/121/2012	A1/12	2.2.1.2	Backyard	JQ858483	6	36hr	2.73	2.6
6	A/Chicken/Egypt/123AS/2012	AS3/12	2.2.1.2	Farm	KJ522708	6	36hr	2.77	3.9
7	A/Chicken/Egypt/128S/2012	S8/12	2.2.1.2	Backyard	JQ858485	6	24hr	2.90	3.4
8	A/Turkey/Egypt/1438S/2014	S38/14	2.2.1.2	Backyard	KP209290	6	24hr	2.73	5.7
9	A/Duck/Egypt/154FAOFL/2015	FL4/15	2.2.1.2	Farm	KR002646	6	36hr	2.90	3.4

* Mean death time on ECE after inoculation in the allantoic cavity of 9–11 day ECE.
** Intravenous pathogenicity index in 6-week-old chickens.
*** Virus excretion in oropharyngeal swabs collected 36 h post infection expressed as log mean embryo infective dose (log10 EID50).