Case Report

Isolation and identification of HPAI from fertile egg derived from broiler breeder farm after natural vaccination breakage during 2010 in Egypt.

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Abstract

In May 2010, during routine monitoring of a commercial broiler breeder flock consists of 6 horizontal houses with total capacity 125000 bird. The farm were vaccinated 3 times with an inactivated H5N1 vaccine. The faerm added under weekly observation and monitoring for continuous 5 weeks after isolation and identification HPAI H5N1 virus from cloacal and oropharyngeal swabs. Different 10 cloacal, oropharyngeal swabs and fertile eggs "after separation of shell, albumin and yolk" samples were directed for AI common gene detection using RTq-PCR test and virus isolation using 10 days old SPF ECE in pooled samples (5 samples/ pool). Moreover 20 blood samples were collected for H5 antibody detection using HI test.

AI H5N1 virus were detected and isolated from different egg parts (egg shell, egg albumin and egg yolk) from the different 6 houses since 2nd weeks post natural infection and stopped after 7 weeks post infection in case of house 5 and 6. The mortality rate was range (27 to 43%) and the drops in egg quality and egg production (20 to 41%) were also recorded. In addition, the HI titers were 3.5 and 5.6 log2 during first 2 weeks of infection and reach to 10log 2 at 4th week post infection. The isolated H5N1 virus from fertile eggs showed high nucleotides and amino-acid identities to recently variant HPAI H5N1 subclade 2.2.1 viruses. The vaccinal break seems to be associated with immune escape mutants and/or improper vaccination. The role of contaminated eggs as a source of infection and as a vehicle for spread of the virus should be considered in area with avian influenza outbreaks, possibilities of vertical transmission weren't studied during this study.

Key words: "Vaccine, "HPAI" Highly Pathogenic Avian Influenza, "AI" Avian Influenza, Egypt, broiler breeder and fertile egg".

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 virus infection is a devastating viral disease causing severe losses in the poultry industry worldwide. AIV are segmented negative-sense RNA viruses belong to the family Orthomyxoviridae that are divided into five genera, including influenza types A, B, and C, Isavirus and Thogotovirus **Palese and Shaw, (2007).** Based on the hemagglutinin (HA) and neuraminidase (NA), two main surface transmembrane glycoproteins, there are at least 17 HA (denoted H1 to H17) and probably 10 NA subtypes (denoted N1–N10) **Tonga *et al.* (2012)**.

The main control measures in the face of an AI outbreak are continuous massive surveillance, proper hygienic culling of infected birds, restriction of movement, and enforcement of biosecurity **Tian *et al.* (2005) and Capua and**

Alexander, (2006). But in case of developing countries, implementation of massive vaccination policy using H5-based inactivated vaccines as supportive control tool was implemented in several countries. Since the incursion of HPAI H5N1 in Egypt in mid-February 2006 **Aly *et al.* (2007); Kilany, (2007) and Safwat, (2007)**, Egypt have been embarked on mass uncontrolled vaccination program for commercial poultry with different types of inactivated H5N1 and H5N2 vaccines with wide variation in standards was implemented as the main control practice **Abdelwhab and Hafez, (2011)**. However, since 2007, infection of vaccinated flocks has increased and subsequent depopulation of positive flocks has not been uncommon **Aly *et al.* (2008)**. By late 2007, H5N1 outbreaks in vaccinated poultry in Egypt have been dramatically increased which criticized the effectiveness of the implemented vaccines **Peyre *et al.* (2009)**. Genetic and antigenic analysis of the circulating field viruses in vaccinated flocks revealed subtle antigenic variation and antigenic drift of the virus in comparison to vaccine strains **Arafa *et al.* (2010a,b); Hafez *et al.* (2010); Abdel-Moneim *et al.* (2011a,b); Abdelwhab *et al.* (2011); Grund *et al.* (2011); Abdelwhab *et al.* (2012)**. These mutations allowed the virus to evade the repertoire of host immune response after vaccination**.**

It was thought that due to the short clinical course of HPAI infected chickens as they usually die within 1–3 days, so it is unlikely to disseminate the HPAI virus in produced eggs via infected chickens and couldn't be detected. But a lot of studies were able to detect AIV in eggs of naturally as well as experimentally infected birds have been previously described **Bean *et al.* (1985): Cappucci *et al,* (1985); Kilany *et al.* (2011) and Abdelwhab *et al.* (2012).**

Also in this study, we studied the possibilities to isolate HPAI H5N1 virus from fertile eggs obtained from naturally infected vaccinated broiler breeder chickens in Egypt during May 2010.

Materials and Methods.

Case report .

During May 2010, Broiler breeder flock consists of 125,000 divided on 6 different poultry houses, with same breed "Hubbard" and same age 32 week, was suffering from HPAI two weeks before starting the sampling due to late reporting. The farm was vaccinated 3 times with H5N1 vaccine seeded by A/Goose/ Guangdong/1/1996/H5N1 (Re-1, Harbin, China) as following (1st vaccination at 10 days old, 2nd after one month and 3rd vaccination at 13 weeks old). The diseased birds were showed cyanosis comb and wattles as well as hemorrhages on the shank, facial edema and nervous manifestation at late stage of infection since 5th week post infection. The drop in egg production in case of HPAI is transient and was ranged from 20 to 41%, and an increase of the number of misshaped eggs (depigmentations, soft eggshell, shell-less and rough eggs). At the same time, mortality percent range from 27 to 43% **(Chart 2).** Samples for laboratory investigation were from each house, 10 tracheal swabs, 10 cloacal swabs, and 10 shelled eggs were collected randomly at weekly basis 14 days after the onset of clinical signs. At the same time, 20 serum samples were collected from each house. For further laboratory investigation, 5 swabs were pooled according to **OIE, (2009)**. From the collected eggs, eggshell wash, albumin, and yolk materials were separated, and each part was tested in 5 pool protocol.

Virus isolation and detection.

Virus isolation carried out using 10-day-old specific-pathogen-free (SPF) hatching eggs via allantoic sac inoculation, **OIE, (2009)**. The HPAI H5N1 virus was isolated after first passage from tracheal and cloacal swabs collected from each house on weekly basis and confirmed by real-time reverse transcription– polymerase chain reaction (RT-qPCR). the HPAI H5N1 was recorded in house 5 and house 6 since 1st week of examination then the virus have been recorded in house 3, house 4, and in case of house 2 and house 1 detection of AI

was after 3rd week of examination. The AIV disease remain in the farm for more than 7 weeks post natural infection, also we record presence of H5 virus in different egg parts including egg shell, egg albumin and egg yolk in different houses and the dissemination of AIV virus in egg content start from 2nd week post natural infection and stops after 5th weeks post infection in case of house 5 and 6 **(Table1, 2 and 3).**

RT-qPCR for H5N1 VIRAL RNA detection. RNA was extracted from 5 sample pools using a MagNA Pure LC Total Nucleic Acid Extraction kit following manufacturer's instructions and a MagNA Pure LC instrument (Roche, Mannheim, Germany). The matrix gene of avian influenza type A viruses was amplified from RNA samples using a one-step Real-Time PCR Kit (Qiagen, Valencia, CA) as described by **(Spackman *et al.*, 2002),** and the test was conducted in a Stratagene MX3005P real-time PCR machine (Stratagene, Agilent Technologies, Santa Clara, CA). Detection of H5N1 subtype was done by avian influenza virus H5N1 Real-Time RT-PCR Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions, using the Light Cycler H 2.0 machine (Roche, Mannheim, Germany). The H5N1 viral RNA could be detected in cloacal and tracheal swabs as well as from eggshell, egg albumin and yolk materials collected from affected houses.

Serological investigation. The detection of avian influenza H5– specific antibodies in the serum samples using hemagglutination inhibition (HI) test was performed according to **OIE, (2009)** using 4 hemagglutinin units of homologous A/Goose/ Guandong/1/1996 H5N1 antigen, supplied by the vaccine-producing company. Serologic examination showed the HI titers ranging from 3.5 to 5.6 log2 during 1st week of monitoring in house 6 and house 5. On the other hand, on other houses 3, 4, 2 and 1 was not tested at first week; moreover HI titers reach to 10 log2 in all houses after 3rd week of examination **(Table 2).**

Sequence and phylogentic analysis. Sequencing of the H5 gene of the virus isolated from cloacal, oropharyngeal swabs and designated as A/chicken/Egypt/10127s-NLQP/2010 (H5N1) was conducted as previously mentioned by **Arafa *et al.* (2010a)** using BigDye Terminator v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI-3130; Applied Biosystems, Foster City, CA). Sequence data were analyzed and edited with BioEdit version 7.0.9.0 **Hall, (1999).** Amino-acid sequences were deduced and a BLASTN search was performed to identify the query sequence and to find similar sequences. The H5 gene sequence of the isolated virus in this study was submitted to GenBank, and the accession number is HQ198274. Phylogenetic analyses for the full length of the H5 genes and the highest homologous H5 gene sequences obtained after blast were produced based on CLUSTAL V multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software (Madison, WI) to determine nucleotide and amino acid sequence similarities and relationships, with 100 bootstrap using a GTR+G model as recommended by ModelTest (Topali). The tree was drawn with Dendroscope **Huson *et al.* (2007).**

The virus had polybasic cleavage motif PQGERRRKKRGLF, which indicates high pathogenic subtype as for clade 2.2.1 viruses. The isolated virus showed 99.8% and 99.7% nucleotides and amino-acid identities, respectively, and clustered mainly with viruses from recently confirmed H5N1 variant viruses and with currently circulating viruses in commercial birds and vaccinated commercial farms from different places in Egypt in 2010.

Table 1. Results of laboratory investigation in house 1 and 2.

Samples type	hs1					hs2				
	W1	W2	W3	W4	W5	W1	W2	W3	W4	W5
Virus isolation										
Tracheal swab	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Post
Cloacal swab	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Post
Egg shell	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Post
Egg albumin	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Neg
Egg yolk	NT	Neg	Neg	Neg	Neg	NT	Neg	Neg	Neg	Post
RT-Qpcr										
Tracheal swab	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Post
Cloacal swab	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Post
Egg shell	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Post
Egg albumin	NT	Neg	Neg	Post	Post	NT	Neg	Neg	Post	Neg
Egg yolk	NT	Neg	Neg	Neg	Neg	NT	Neg	Neg	Neg	Post
Serology HI test										
HI mean titer	NT	8.5	9	10	10	NT	8.3	9.5	10	10
STD ±	NT	0.46	0.3	0.24	0.2	NT	0.9	0.76	0.32	0.4
Post %	NT	100%	100%	100%	100%	NT	100%	100%	100%	100%
Mortality	2%	2.6%	10%	15.8%	23%	1.2%	4%	11%	21%	27%
Egg production in comparison with standard curve										
	88%	89%	70%	65%	54%	89%	92%	74%	53%	56%

hs= poultry house NT= Not tested

Table 2. Results of laboratory investigation in house 3 and 4.

Samples type	hs3					hs4				
	W1	W2	W3	W4	W5	W1	W2	W3	W4	W5
Virus isolation										
Tracheal swab	NT	Post	Post	Post	Post	NT	Post	Post	Post	Post
Cloacal swab	NT	Post	Post	Post	Post	NT	Post	Post	Post	Post
Egg shell	NT	Post	Post	Post	Post	NT	Post	Post	Post	Post
Egg albumin	NT	Post	Post	Post	Neg	NT	Neg	Post	Post	Neg
Egg yolk	NT	Neg	Post	Post	Neg	NT	Neg	Post	Post	Post
RRT-PCR										
Tracheal swab	NT	Post	Post	Post	Post	NT	Post	Post	Post	Post
Cloacal swab	NT	Post	Post	Post	Post	NT	Post	Post	Post	Post
Egg shell	NT	Post	Post	Post	Post	NT	Post	Post	Post	Post
Egg albumin	NT	Post	Post	Post	Neg	NT	Neg	Post	Post	Neg
Egg yolk	NT	Neg	Post	Post	Neg	NT	Neg	Post	Post	Post
Serology HI test										
HI mean titer	NT	6.5	9.3	10	10	NT	7.6	9.2	10	10
STD ±	NT	0.35	0.87	0.12	0.1	NT	1.2	1.3	0.2	0.1
Post %	NT	100%	100%	100%	100%	NT	100%	100%	100%	100%
Mortality	8%	9.6%	13%	25.8%	34%	6.3%	9.9%	15%	23%	34%
Egg production in comparison with standard curve										
	75%	75%	65%	64%	53%	75%	73%	64%	53%	59%

hs= poultry house NT= Not tested

Table 3. Results of laboratory investigation In house 5 and 6.

Samples type	hs5					hs6				
	W1	W2	W3	W4	W5	W1	W2	W3	W4	W5
Virus isolation										
Tracheal swab	Post	Post	Post	Post	Neg	Post	Post	Post	Post	Neg
Cloacal swab	Post	Post	Post	Post	Neg	Post	Post	Post	Post	Neg
Egg shell	Post	Post	Post	Post	Neg	Post	Post	Post	Post	Neg
Egg albumin	Post	Post	Post	Post	Neg	Neg	Neg	Post	Post	Neg
Egg yolk	Neg	Neg	Post	Post	Neg	Neg	Neg	Post	Post	Neg
RRT-PCR										
Tracheal swab	Post	Post	Post	Post	Neg	Post	Post	Post	Post	Neg
Cloacal swab	Post	Post	Post	Post	Neg	Post	Post	Post	Post	Neg
Egg shell	Post	Post	Post	Post	Neg	Post	Post	Post	Post	Neg
Egg albumin	Post	Post	Post	Post	Neg	Neg	Neg	Post	Post	Neg
Egg yolk	Neg	Neg	Post	Post	Neg	Neg	Neg	Post	Post	Neg
Serology HI test										
HI mean titer	5.6	6.5	10	10	10	3.5	7.6	10	10	10
STD ±	2.2	1.35	0.87	0	0	2.6	2.2	0	0	0
Post %	80%	100 %	100 %	100%	100%	60%	100%	100%	100%	100%
Mortality	17%	23.5 %	29%	37%	38%	18%	22.2%	27.9%	38%	43%
Egg production in comparison with standard curve										
	65%	53%	65%	68%	75%	55%	59%	64%	74%	80%

hs= poultry house NT= Not tested

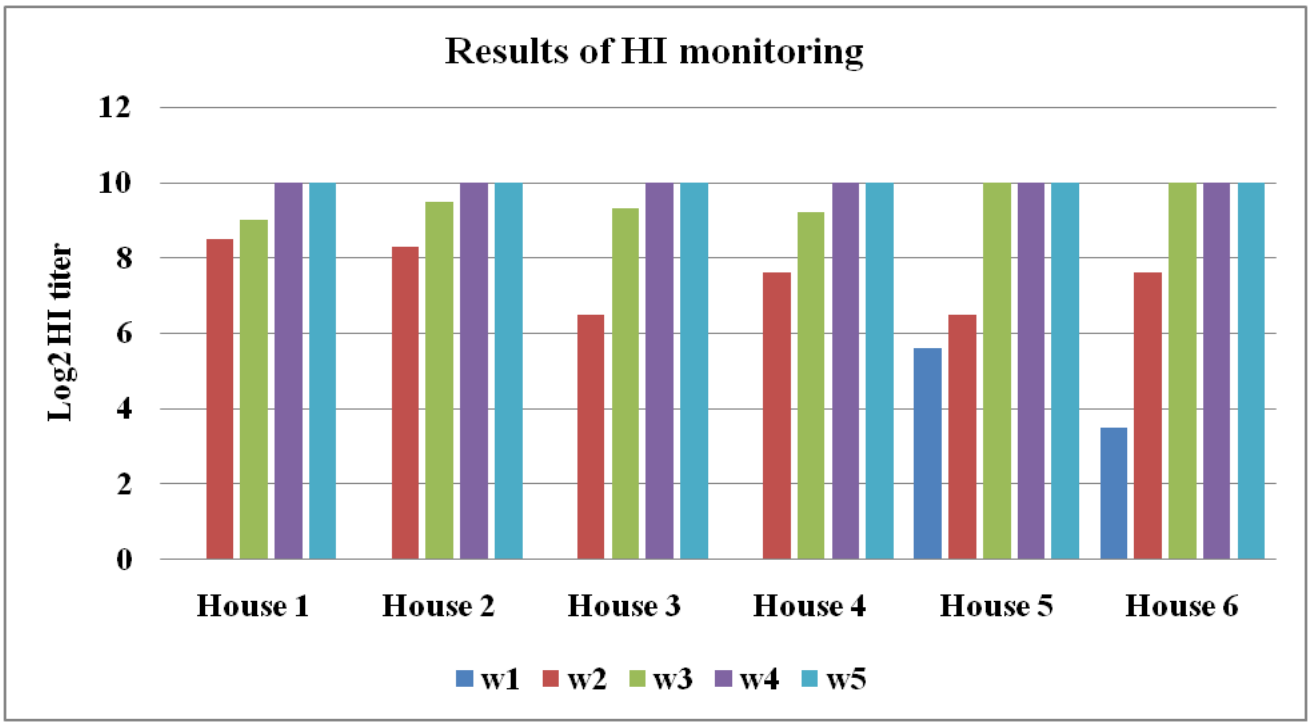

Chart 1. Results of HI testing during the 5 tested weeks.

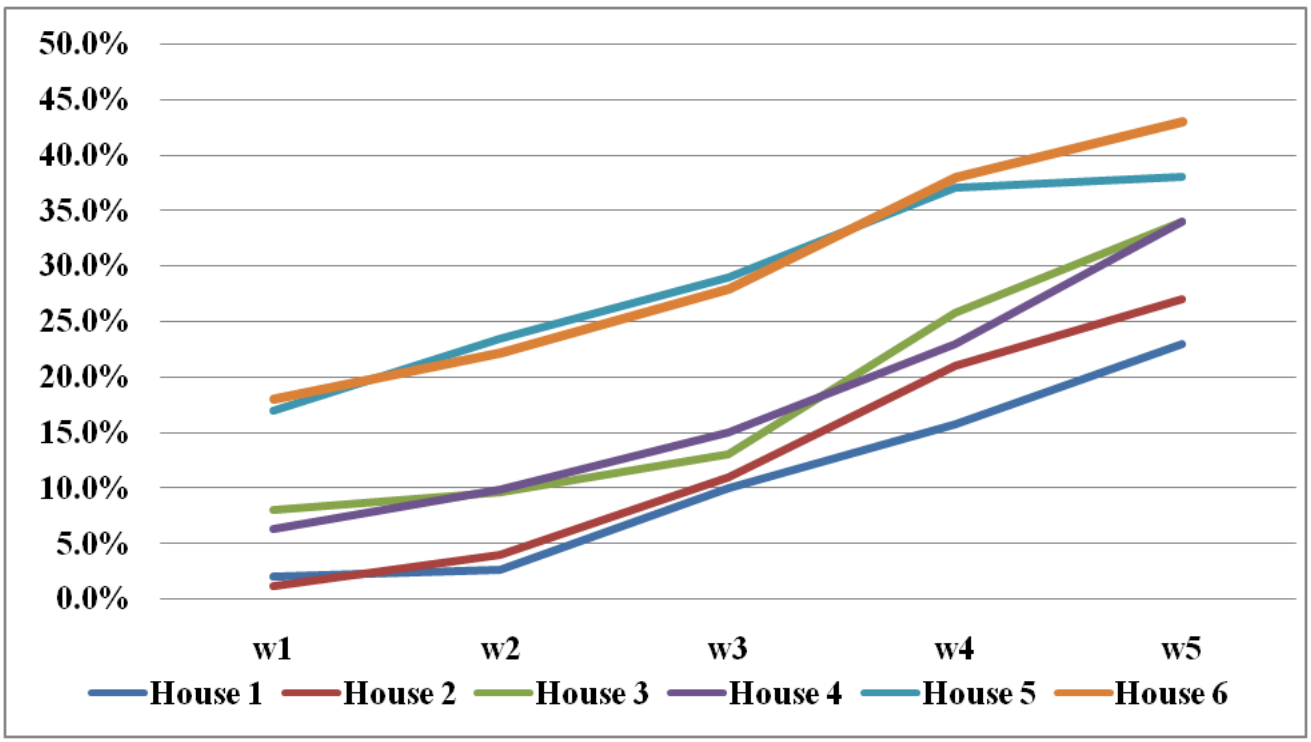

Chart 2. Cumulative Mortality.

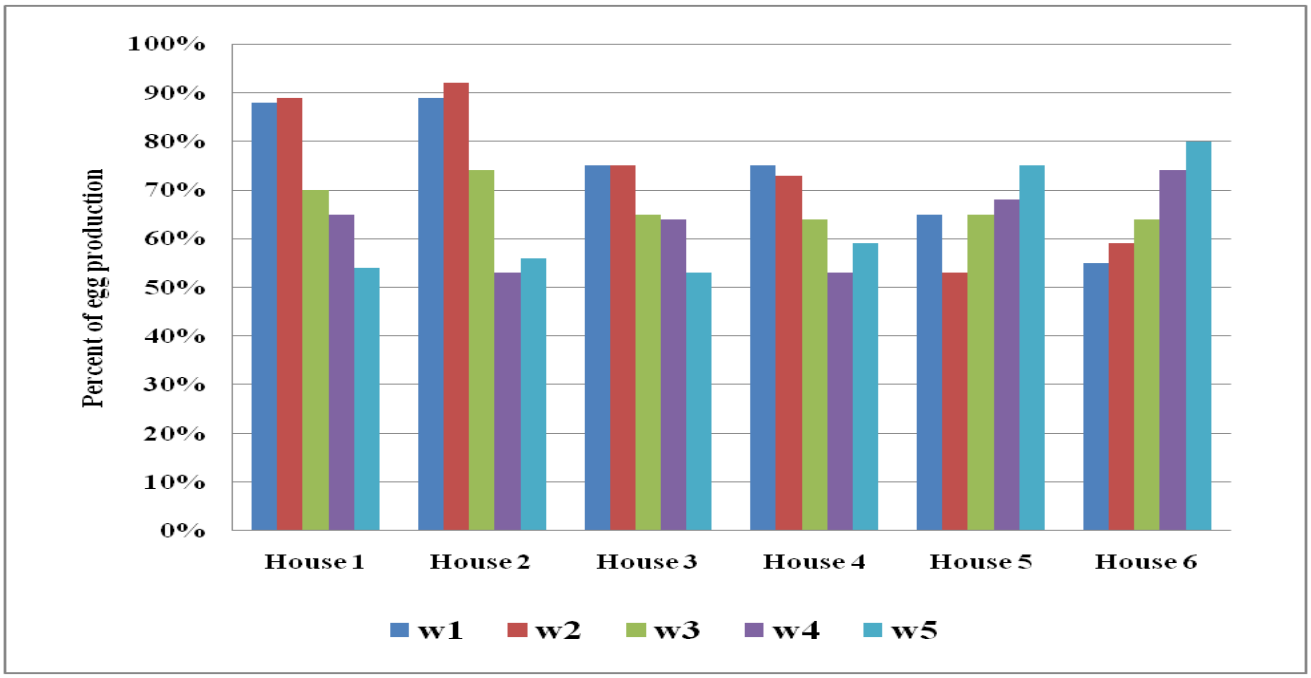

Chart 3. Drop in egg production curve during 5 weeks of examination

Discussion.

HPAI H5N1 outbreaks in vaccinated poultry flocks have been reported in many countries and there were many factors are incriminated as a cause of the vaccinal breaks, such as incomplete immunization coverage and/or vaccine failure, as described by **Simth *et al.* (2006)** in China, **Aly *et al.* (2008)** in Egypt and **Henning *et al.* (2009)** in Vietnam. The Continuous emerging of the endemic HPAI H5N1 virus within vaccinated birds in Egypt in the last five years and emerging of different antigenic and genetic mutant viruses as in group B (B1, B2.1 and B2.2) **Kilany, (2013)**. So far, two different viruses are circulating now in Egypt; subgroup A2 "classic 2.2.1" strains infect mostly backyard birds and human and the newly emerging subgroup B2.2 "2.2.1 variants" circulate mainly in vaccinated commercial farms in Egypt, circulation of genetically and antigenically distinct H5N1 viruses in vaccinated poultry flocks is common **Abdelwhab *et al.* (2010); Arafa *et al.* (2010 a, b); Balish *et al.* (2010), Abdelwhab *et al.* (2011) and Kilany *et al.* (2011).**

The virus RNA were detected from eggshell, albumin and egg yolk by RT-q PCR, and the isolation of the virus was successful from egg shell, egg albumin and yolk as shown in **(Table 1, 2 and 3)** which is in accordance with recently reported results **Pillai, *et al.* (2010)** from experimentally infected turkey by H3N2 AIV, moreover **Bean, *et al.* (1985); Cappucci *et al.* (1985); Li *et al.* (2006); Kilany *et al.* (2010); Abdelwhab *et al.* (2012)** isolate AIV from internal contents of eggs obtained from naturally or experimentally infected birds. Also, HPAI H5N1 virus was recovered from the yolk and albumin of naturally infected Japanese quail **Promkuntod *et al,* (2006)** and egg shells of ducks and geese **Li *et al.* (2006).**

The role of contaminated eggs as a source of infection and as a vehicle for spread of the virus should be considered in area with avian influenza outbreaks **Kilany *et al.* (2010); Pillai *et al.* (2010); Abdelwhab *et al.* (2011)**. However proof of vertical transmission is lacking. Moreover, HPAIV is usually embryo lethal and

hatching of internally contaminated eggs is unlikely **Easterday *et al.* (1997)**.

The mortality rate during this study reach (27 to 43%) and egg production and egg quality dropped (20 to 41%). On the other hand, serologic examination of sera collected two weeks after the onset of the clinical signs, the HI titers in the first affected two houses (House 6 and 5) were 5.6 and 3.5 log2, which indicate improper vaccination **Tian *et al.* (2005)** and the HI titers shows repaid poster since week 3 post infection in all houses and reach to 10 log 2, using the homologous H5N1 antigen.

The isolated virus had polybasic cleavage motif ERRRKKR/GLF, which indicates high pathogenic subtype as for clade 2.2.1 viruses **Abdelwhab, (2012): Kilany, (2013)**. It has relatively high similarities to nucleotides and amino acids and clustered with viruses from recently confirmed HPAI H5N1 commercial poultry cases in Egypt. Moreover, several amino-acid substitutions of viral H5 protein were observed at different positions not shown in this study. These amino-acid residues are located mainly in the most exposed part of the globular head of the hemagglutinin **Kilany, (2013),** In Egypt, circulation of genetically and antigenically distinct H5N1 viruses in vaccinated poultry flocks is not uncommon **Arafa *et al.* (2010 a, b); Balish *et al.* (2010), Abdelwhab *et al.* (2011) and Kilany *et al.* (2011).**

Accordingly, in case of an outbreak of HPAI H5N1, regular disinfection of contaminated eggshell alone will be insufficient to disclose the potential hazard for spread of the virus through infected, contaminated cracked eggs, and egg flats during their marketing or from one farm to another by vendors and vehicles. Therefore, we recommend that eggs from infected birds should be culled in the farm to prevent further spread of the virus into the environment. In conclusion, although currently no available data suggest that the vertical transmission of HPAI but handling of contaminated eggs could be potential hazards of spreading AI infection to the surrounding environment by movement of eggs and contaminated egg trays in areas of an outbreak must be emphasized.

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