

## Molecular Detection of H5N1, H9N2 and Newcastle Disease Viruses Isolated from Chicken in Mixed Infection in Egypt

<sup>1</sup>Kh. M. Elbayoumi, <sup>1</sup>K.M. Mahgoub, <sup>1</sup>Hoda M. Mekky, <sup>1</sup>Eman R. Hassan,  
<sup>1</sup>Zeinab M.S. Amin Girh, <sup>1</sup>Asmaa M. Maatouq, <sup>2</sup>Hanaa A. El-Samadony,  
<sup>1</sup>Nagwa S. Rabie, <sup>3</sup>M.A.A. Ali and <sup>1</sup>M.A. Kutkat

<sup>1</sup>Department of Poultry Diseases, Veterinary Research Division,  
National Research Centre, Dokki, Giza, Egypt  
<sup>2</sup>Animal Health Research Institute, Giza, Egypt  
<sup>3</sup>National Research Centre, Dokki, Giza, Egypt

**Abstract:** Total of 335 fecal and nasal swabs were collected from Broilers, Broiler Breeders, Layers and duck flocks from farms and backyard in different Provinces in the period from 2011 to the year 2013. All these flocks had history for previous vaccination against avian influenza and Newcastle disease viruses. All samples were inoculated in 9-day old specific pathogenic free (SPF) embryonated chicken eggs through allantoic sac. Rapid haemagglutination (HA) test was done for detection of haemagglutinating viruses. Results of Haemagglutination revealed that, 12 (3.6%) out of 335 collected samples were positive by using HA test. The results revealed that 4 (4.4%) out of 90 broiler samples from Fayoum Governorate were positive by HA test. One sample (6.6%) out of 15 samples; 2 samples (1.3%) out of 157 samples and 2 (3.4%) out of 58 were positive by HA test in broiler, breeder and layer flocks respectively from Sharkia Governorate. In Menofia Governorate, one sample (7.7%) out of 13 from backyard and 2 samples (100%) out of 2 collected from ducks were positive by HA test. HA positive samples either Newcastle disease and/or avian influenza virus. Results of virus detection by RT-PCR using F gene primers proved that all isolated viruses were ND negative. Also, the results of RT-PCR test for H5 and H9 genes proved that AIV H5N1 virus was detected in 8 isolates (66.6%) out of 12 HA positive samples, where 5 samples (41.6%) from Sharkia and 3 samples (25%) from Menofia. Also, AIV H9N2 was detected in 9 isolates (75%) out of 12 HA positive samples, where 5 (41.6%) samples from Sharkia and 4 samples (20%) from Fayoum.

**Key words:** Influenza • Molecular Detection • Vaccine • Avian • Control • Newcastle Disease

### INTRODUCTION

Avian Influenza virus (AIV) has been isolated from different outbreaks in poultry species causing severe economic losses all over the world. First outbreak of avian influenza in Egypt was reported in February 2006, the disease infected many poultry farms in more than 18 governorates more rapidly, moreover the virus proved to be transmitted from infected birds to human being (Zoonotic disease) recording about 18 infected cases at the end of 2006 with 10 deaths cases and resulted in severe economic losses represented as mass loss in

Egyptian poultry flocks as well as sharp reduction in egg production in both table and hatched eggs.

The disease in chicken varied according to strain either low pathogenic avian influenza virus (LPAIV) as those contains H9 or H7 antigen or the highly pathogenic avian influenza virus (HPAIV) as those contains H5 antigen. Infection with low pathogenic avian influenza virus resulting in no detectable signs, decrease in egg production and or upper respiratory signs [1] more over if infection with low pathogenic avian influenza associated with secondary pathogens leads to severe morbidity and mortality occurs [2] as complication with

**Corresponding Author:** M.A. Kutkat, Veterinary Research Division, National Research Centre,  
Postal Code: 12622, Dokki, Giza, Egypt.

Newcastle disease (ND) virus [3]. On the other hand HPAIV H5N1 strains cause more severe clinical signs with high mortality [4] as typical clinical signs of HPAIV included cyanotic combs and wattles, subcutaneous hemorrhages in the unfeathered skin, edema of the face and limbs as well as gasping [5]. In Egypt outbreaks of avian influenza virus waves are mainly three: one at 2006, the second at 2007 and third in 2008-2009 as this 3<sup>rd</sup> outbreak was recognized with more mutation in nucleotide and amino acid sequences that assist the superior effect and insistence need of autogenous commercial local vaccine.

The present study was aimed to explore the complicated infection with H5N1 and H9N2 as well as Newcastle disease viruses in poultry farms by using of RT-PCR at the period from 2011 to the year 2013.

### MATERIALS AND METHODS

**Samples Collection:** Cloacal and tracheal swabs were collected from 335 Broilers, Broiler Breeders, Layers and duck farms and backyard in different Governorates (Fayoum, Sharkia and Menofia) as shown in Table (1). All these flocks had history for previous vaccination against Newcastle disease viruses (NDV) and against AIV with H5N2 or H5N1 inactivated oil emulsion vaccine. All swabs were collected in Dulbecco's minimum essential medium containing antibiotic-antimycotic mixture (Penicillin 10,000 IU/ml, Streptomycin 10,000 µg/ml and Amphotericin B 250 µg/ml). After collection, the samples were transported to the laboratory on ice and were stored in the laboratory at -80°C until tested.

**Detection of HA Positive Samples:** Virus isolation was performed in SPF embryonated chicken's eggs (Obtained from Kom-Oshim Company, El-Fayoum Governorate, Egypt). In accordance with the OIE guidelines [6] tubes containing cloacal or tracheal swabs were vortexed to ensure thorough mixing. Swabs were routinely processed individually. According to the method of Sunchai Payungporn *et al.* [7], Supernatant fluid was inoculated via the allantoic route into 9 day-old embryonated SPF chicken eggs with 200 µl sample per egg. The inoculated eggs were incubated at 37°C and candled daily for 7 days.

At the end of the incubation period, allantoic fluid was harvested from the inoculated eggs and assayed for haemagglutination (HA) activity by the "rapid HA test" using 10% washed chicken red blood cells according to Swayne *et al.* [8]. The HA-negative samples were passaged once more in SPF embryonated chicken eggs. All HA-positive allantoic fluids were further tested by reverse transcription-polymerase chain reaction (RT-PCR) for NDV and AIV subtypes H5 and H9.

**RNA Extraction:** The extraction of viral RNA was conducted from all HA-positive allantoic fluid using QIAamp Viral RNA Mini Kit (Qiagen, Germany) (Qiagen®, Germany Catalog No. 52904) following the manufacturer's instructions. Finally, the elution buffer containing RNA were stored at -80°C.

**RT-PCR Amplification and Gel Electrophoresis:** Extracted RNA was tested for the presence of NDV and AIV subtypes H5 and H9 by Reverse transcription-polymerase chain reaction (RT-PCR) assays using specific primers as shown in Table (2).

Table 1: Number of Samples which collected from different localities

Type of chicken	Fayoum *Ex. No.	Sharkia Ex. No.	Menofia Ex. No.	Total
Broiler	90	15	---	105
Breeder	---	157	---	157
Layer	---	58	---	58
Backyard	---	---	13	13
Duck	---	---	2	2
Total	90	230	15	335

\* Ex. No. = Examined Number

Table 2: Oligonucleotide primers for detection of NDV and AIV subtypes H5 and H9 by reverse transcription polymerase chain reaction (RT-PCR)

Target gene fragment	Primer name	Primer sequence	Amplicon size (bp)	Reference
F (ND)	NDV-F328	5'-CCT TGG TGA ITC TAT CCG IAG G-3'	254	Seal <i>et al.</i> [9]
	NDV-R581	5'-CTG CCA CTG CTA GTT GIG ATA TAC C-3'		
H5 (AI)	H5-kha-1	5'-CCT CCA GAR TAT GCM TAY AAA ATT GTC-3'	311	Slomka <i>et al.</i> [10]
	H5-kha-3	5'-TAC CAA CCG TCT ACC ATK CCY TG -3'		
H9 (AI)	H9-151f	5'-CTY CAC ACA GAR CAC AAT GG-3'	488	Lee <i>et al.</i> [11]
	H9-638r	5'-GTC ACA CTT GTT GTR TC-3'		

Amplification was performed on the basis of one-step protocols using appropriate RT-PCR Kits (Qiagen catalog No. 210212) according to the manufacturers' instructions.

**RT-PCR Temperature Conditions:** The PCR condition for the amplification of NDV was performed with the following programme: RT at 48°C for 45 min., one cycle at 94°C for 2 min., 40 PCR cycle at 94°C for 30 s (Denaturation), 60°C for 1 min. (Annealing), 68°C for 2 min. (Extension) and one cycle of final extension step at 68°C for 7 min. in automated thermal cyclers.

The previously extracted RNAs of negatively NDV were used for identification of AIV subtypes H5 and H9. For amplification of subtype H5 of AIV, a different programme was followed, RT at 50°C for 30 min., one cycle at 95°C for 15 min., 40 PCR cycle at 94°C for 30 s (Denaturation), 58°C for 30 s (Annealing), 68°C for 2 min. (Extension) and one cycle of final extension step at 72°C for 10 min. in automated thermal cyclers.

The steps for amplification of subtype H9 was the same as H5 but with different annealing temperature (50 °C) and extension temperature and time (72 °C for 1 min).

The reaction's products will be analyzed by electrophoresis on a 2 % agarose gel stained with ethidium bromide visualized under ultraviolet light for image capture.

## RESULTS

**Field Examination:** Symptoms was varied from sudden death in ducks to severe clinical signs such as cyanosis of comb and wattles, nervous signs, facial edema,

respiratory signs and diarrhea, in addition to decrease in egg production and low egg shell quality in vaccinated flocks of Layers breeders and duck flocks.

At necropsy, subcutaneous hemorrhages of thigh muscles, congestion in internal organs in broiler in addition to egg peritonitis and inflammation of the ovary (ovaritis) in layer and breeder flocks were observed.

**Results of Haemagglutination Test:** As shown in Table (3), 12 (3.6%) out of 335 collected samples were positive by using HA test. The results revealed that 4 (4.4%) out of 90 broiler samples from Fayoum Governorate were positive by HA test. One sample (6.6%) out of 15 samples, 2 samples (1.3%) out of 157 samples and 2 (3.4%) out of 58 were positive by HA test in broiler, breeder and layer flocks respectively from Sharkia Governorate. In Menofia Governorate, one sample (7.7%) out of 13 from backyard and 2 samples (100%) out of 2 collected from ducks were positive by HA test. HA positive samples suspected to be ND and/or AIV.

**Results of Virus Detection by RT-PCR:** RT-PCR using F gene primers proved that all isolated viruses were ND negative. Also, the results of RT-PCR test for H5 and H9 genes proved that AIV H5N1 virus was detected in 8 isolates (66.6%) out of 12 HA positive samples where 5 samples (41.6%) from Sharkia Governorate and 3 samples (25%) from Menofia Governorate. Also, AIV H9N2 was detected in 9 isolates (75%) out of 12 HA positive samples where 5 samples (41.6%) from Sharkia Governorate and 4 samples (20%) from Fayoum Governorate as shown in Table (4).

Table 3: Results of Haemagglutination (HA) test:

Governorates	Total Samples	Types of birds										Total HA +ve
		Broiler		Breeder		Layer		Backyard		Duck		
		*Ex. No.	HA +ve	Ex. No.	HA +ve	Ex. No.	HA +ve	Ex. No.	HA +ve	Ex. No.	HA +ve	
Fayoum	90	90	4 (4.4%)	-	-	-	-	-	-	-	-	4/90
Sharkia	230	15	1 (6.6%)	157	2 (1.3%)	58	2 (3.4%)	-	-	-	-	5/230
Menofia	15	-	-	-	-	-	-	13	1 (7.7%)	2	2 (100%)	3/15
Total	335		5		2		2		1		2	12/335 (3.6%)

\* Ex. No. = Examined Number

Table 4: Results of RT-PCR

Governorates	No. of PCR +ve to NDV	No. of PCR +ve to AIV H5N1	No. of PCR +ve to AIV H9N2
Fayoum	-	-	4 (20%)
Sharkia	-	5 (41.6%)	5 (41.6%)
Menofia	-	3 (25%)	-
Total	-	8 (66.6%)	9 (75%)

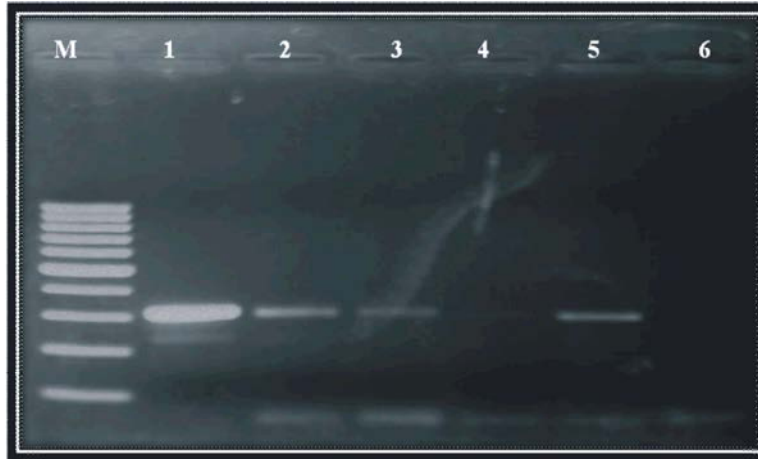


Fig. 1: H5 Subtyping of avian influenza viruses

Agarose gel electrophoresis of RT-PCR Amplification product of approximately 320 bp molecular weight of the H5 gene.

Lane (1): Positive control samples

Lane (2 to 5): positive samples.

Lane (6): negative samples

Lane M: 100 bp DNA ladder

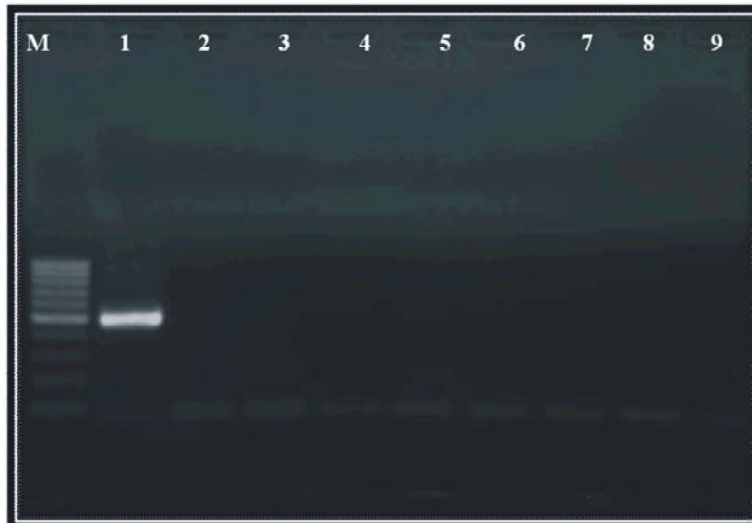


Fig. 2: H9 Subtyping of avian influenza viruses

Agarose gel electrophoresis of RT-PCR Amplification product of approximately 488 bp molecular weight of the H9 gene.

Lane (1): Positive sample

Lane (2 to 8): negative samples

Lane (9): negative control

Lane M: 100 bp DNA ladder

To confirm the identity subtyping of the causative virus of avian influenza which circulating in Egypt. RNA extracted from positive Haemagglutinating (HA) samples were tested by RT-PCR using specific primers for amplifying partial sequence of H5 and H9. Results

demonstrated that, the amplification was appeared at 320 bp for H5 and 488 bp for H9 corresponding to the expected molecular weights of HA genes from the locations of the used primers as shown in Fig. (1) and Fig. (2).

## DISCUSSION

Avian influenza (AI) H5N1 virus strains are of worldwide circulation in birds, responsible for the current severe outbreaks in poultry and other birds. Human, feline and other mammals in Asia, Africa, Europe and USA [12, 13]. World human and animal health authorities (WHO, OIE and FAO) considered AI H5N1 as a notifiable disease required international cooperation on the scientific, information and economical to compact such infection and avoid the possible human pandemic.

In Egypt, AI was under focus as enzootic cases of fowl plague had been reported 1923-1945 [14, 15].

In mid February 2006 outbreaks of H5N1 had reported in backyard and commercial poultry flocks with human cases. Following the failure in "stamping out" both Chinese (H5N1) and (H5N2), inactivated vaccines beside vaccines other than Chinese were used for prevention of the disease.

By the year of 2009 and start of 2010 H5N1 was reemerged with signs as showed in 2006 in some areas accompanied with nervous signs and mortalities were ranged between 40-80 % with drastic drop in egg production, on the other hand, it was observed that; H5N1 infection was accompanied with complicated chronic respiratory disease (CCRD).

Preliminary field investigations showed that, H5N1 infection may be complicated with other viruses in layer flocks, which vaccinated or non vaccinated against H5N1 and/or H9N2.

Respiratory signs are more prominent and included rales, sneezing and coughing. Other galliforme birds have similar clinical signs but may live longer and have evidence of neurologic disorders such as paresis, paralysis [16]. The occurrence of infection in Menofia followed by Sharkia and other Governorates; may be due to poorly controlling and movement of infected flocks and poor of biosecurity which lead to AI to become endemic in poultry populations.

One of the main problems associated with the use of these vaccines beside the low potency is the delay in immune response which observed in the third week post vaccination. This means that the poultry be unprotected at the first three weeks of their short life. For monitoring AIV infection in the field setting, the use of rapid antigen detection method for primary field screening of both influenza type A and specifically H5 antigen was successful indicator. Our finding was accord with report of Saif *et al.* [17] they reported that, depression is common as are significant declines in feed and water

consumption. Precipitous drops in egg production occur in breeders and layers with typical declines including total cessation of egg production within six days.

In Egypt no available literature dealing the regular monitoring of AI mixed with NDV in Egyptian Governorates, although the prevention of AI infection and spread is based on regular monitoring, hygienic measures, while control of AI is based on eradication, disinfection quarantine and compensation. Prevention of AI passed on strategies as biosecurity to prevent exposure of flocks to the influenza virus, continuous monitoring, reporting of AI suspected and applying control measures, depopulation and disinfection and quarantine of positive cases as a short strategy [18-22]. In endemic area, vaccination of poultry flocks by inactivated became the only solution in the long-term strategy. Vaccination is targeting to lower losses from mortality, reduce the viral load in the environment and risk of human infection as well as eradication of positive cases [19, 23-25].

In our study, positive HA samples suspected to be ND and/or AIV, as shown in symptoms poultry flocks.

Detecting AIV and NDV at low concentrations from tracheal and cloacal swabs was carried out by Ming Jun *et al.* [26], they used a highly sensitive immunological-polymerase chain reaction (Immuno-PCR) method.

In this study, although vaccination in backyard birds with bivalent vaccine against AIV and NDV was occurred, high percentage of infection with influenza viruses (H5N1 and H9N2) in broilers, duck, breeder and layers was observed, this may be due to the lack of hygienic measure and the low potency of the vaccine. We efficiently identified type A of avian influenza and Haemagglutinating virus from naturally infected chickens.

Results of the present study showed that co-infection of H5N1 with H9N2 AIV not only increased the severity of H9N2 AIV clinical signs and gross lesions, but also increased the mortality rate and decrease in egg production, then extended viral shedding period of H9N2 avian influenza virus although ND not detected as co- infection virus, but detected individually. Haghghat Jahromi *et al.* [27] reported that, avian influenza virus of H9N2 subtype is pathotyped as a non-highly pathogenic virus. However, frequent incidences of avian influenza of high mortality that are caused by H9N2 viruses have been observed in broiler chicken farms in Iran and some other Asian countries. Co-infections or environmental factors may be involved in such cases. Infectious microorganisms have been implicating in taking part in the cases of co-infection.

In conclusion avian influenza virus and Newcastle disease virus could be detected only in subclinical cases of breeders and layers and infect some flocks although vaccination was occurred in these flocks, in the other hand, the presence of H9N2 in flocks may be decrease the immune response of birds against most viral disease infection and can cause high mortalities in broilers and decreasing in egg production in layers.

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