



THE ROLE OF AVIAN INFLUENZA VIRUS (SUBTYPES H9 AND H5) AND AVIAN INFECTIOUS BRONCHITIS VIRUS IN AN OUTBREAK AFFECTING COMMERCIAL POULTRY FLOCKS IN EGYPT DURING 2012

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ABSTRACT

Avian influenza virus (AIV) and infectious bronchitis virus (IBV) have been recognized as ones of the most important viral pathogens in poultry. Recently, commercial poultry flocks in Egypt have suffered massive mortalities associated mainly with respiratory manifestations. Subsequently, the involvement of AIV and IBV has been suspected. In this study, tracheal samples from 18 affected chicken flocks during the period of January to March 2012 were tested for AIV H5, H9 and IBV by using one-step reverse transcription PCR (RT-PCR). The positive RT-PCR results were further confirmed by inoculation of filtered tissue homogenates in specific pathogens free (SPF) eggs followed by performing haemagglutination assay on the allantoic fluid. The RT-PCR results showed that 6 flocks were positive for AI subtype H9, two flocks were positive for AI subtype H5, two flocks were positive for IBV, two flocks were positive for both AI subtype H9 and IBV, and 6 flocks were negative for all of these viruses. Our data showed that these viruses could have an important role in mortalities that have occurred in the poultry flocks during the winter of 2012. Further studies are necessary to assess circulating strains and the impact of co-infections of these viruses and other pathogens.

Keywords: avian influenza, infectious bronchitis virus, RT-PCR, mortalities, chicken flocks

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1. INTRODUCTION

In Egypt, poultry industry remains very important and represents a major economic activity. Regarding broiler production alone, more than 300 million broilers are fattened annually throughout the country (Abdelwhab and Hafez, 2011). Lately, the rates of mortalities and different forms of clinical signs, mainly respiratory in nature have been increased in Egyptian commercial chicken flocks. Avian influenza virus (AIV) is believed as one of the main causes of chicken respiratory diseases in Egypt since February 2006 (Saad et al., 2007). Avian influenza (AI) is a respiratory disease in poultry of zoonotic importance caused by influenza A viruses

of the family Orthomyxoviridae. AIVs infecting birds occurs in two forms. The highly pathogenic AI (HPAI) previously known as "fowl plague" causes a severe systemic disease with mortality up to 100%, and the low pathogenic AI (LPAI) usually causes minimal clinical signs other than a slight drop in egg production (Alexander, 2000). Although 16 haemagglutinin (HA) subtypes of AIVs have been described (Fouchier et al., 2005), the HPAI has only been associated with some strains of the H5 or H7 HA subtype (Senne et al., 1996). In Egypt, HPAI H5N1 virus has established an endemic status in Egypt, even in vaccinated poultry (Abdelwhab and Hafez, 2011).

Moreover, H5N1 virus has acquired increased infectivity for humans and can cause 60% mortality (WHO, 2006). In additions, infection of poultry with H9N2 viruses has become ubiquitous and endemic in several countries. By 1997, H9N2 viruses have been isolated in multiple avian species including chickens, ducks, turkeys, quail, geese and pigeons, throughout Asia (Guan *et al.*, 2000), the Middle East, Europe and Africa (Capua and Alexander, 2004) and for the first time from humans in Hong Kong and China, in 1999 (Peiris *et al.*, 1999, Guo *et al.*, 1999, Peiris *et al.*, 2001). Moreover the H9N2 virus was isolated in neighboring countries such as Jordan (Monne *et al.*, 2007), Tunisia (Tombari *et al.*, 2011), Saudi Arabia (Haghighat-Jahromi *et al.*, 2008). H9N2 viruses produce significant disease problems in poultry resulting in great economic losses due to reduced egg production or high mortality with co-infection with other opportunistic pathogens such as avian infectious bronchitis viruses (IBV) (Haghighat-Jahromi *et al.*, 2008). IBV, a member of the Coronaviridae family (order Nidovirales, genus Coronavirus), is a highly contagious pathogen of domestic fowls worldwide. It replicates primarily in the respiratory tract but also in the epithelial cells of the gut, kidney and oviduct (Cavanagh and Naqi, 2001). IBV is endemic in probably all countries that raise chickens. In Egypt, IBV has been detected and many isolates have been obtained from Egyptian poultry farms (Abdel-Moneim *et al.*, 2006). This study was designed to investigate the association between detection of AIV subtypes H5 and H9 and IB viruses and occurrence of mortalities in chicken flocks in Egypt, using one-step reverse transcription-polymerase chain reaction (RT-PCR) technique.

2. MATERIALS AND METHODS

Positive controls for RT-PCR reactions were obtained from commercial IBV vaccine, H9 N2 (A/chicken/Egypt

/VRLCU-2/2012), and H5N1 (A/chicken/Egypt/VRLCU-6/2012).

2.1. Samples:

During the period of January-March 2012, tracheal samples of freshly dead birds from 18 chicken flocks were submitted to Central Virology Diagnostic Laboratory, Faculty of Veterinary Medicine, Sadat city. Each flock represents a separate farm. These farms were located in Giza, Sharkia, and Gharbia governorates. These flocks were of various ages ranging from 29 to 32 days and 35-48 weeks of age for broilers and layers respectively. In the majority of all flocks, mortality rate was of 15-25%.

2.2. Sample preparation:

A pool of tracheas from each flock was homogenized in sterile normal saline in a sterile mortars and pestles. The homogenates were spun at 1500 rpm for 10 minutes. The supernatants were collected and kept at -75 °C until used for RNA extraction and egg inoculation.

2.3. RNA extraction

Extraction of RNA was performed on the supernatant of pooled homogenate from tissues that taken from each flock using a QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's procedure.

2.4. One-step RT-PCR

Five microliters of RNA was used in 25 µl reaction mixtures using the Thermo Scientific Verso One-Step RT-PCR Kit (ABgene[®] UK) with H5, H9, IBV specific primers. The sequence, target gene, expected size and reference of each primer are showed in table 1. The reactions were heated at 50°C for 15 min followed by verso inactivation at 95°C for 2 min then 35 cycles of 95°C for 20 sec, 55°C for 30 sec, 72°C for 30 sec, followed by 72°C for 10 min in case of H5. The thermal profile was the same except annealing temperature in case of H9 and IBV. Annealing temperature of H9 and IBV primers was 50 °C and 48°C respectively. PCR products

were electrophoresed on a 1% agarose gel in 1X Tris–acetate–EDTA (TAE) buffer (40 mM of Tris and 2 mM of EDTA, with a pH value of 8.0) containing of ethidium bromide for 45 min at 100 V, then

visualized and photographed under 304 nm UV light (UV Transilluminator, Major Science).

Table 1: Sequences, expected size products and references of primers used in this study

Target gene	Primer sequence	Amplicon length (bp)	Reference
H5	H5F:5'-GACTCAAATGTCAAGAACCTTTA-3'	189	(Payungporn et al., 2004)
H9	H5R:5' -CCACTTATTCCTCTCTGTTTAG-3'	488	
IBV (S)	H9F: 5'- CTYCACACAGARCACAATGG-3'	464	(Xie et al., 2006)
	H9R: 5'- GTCACACTTGTGTTGTRTC-3'		(Adzhar et al., 1997)
	XCE1: 5' CACTGGTAATTTTTTCAGATGG 3'		
	XCE2: 5' CTCTATAAACACCCTTACA 3'		

2.5. Egg inoculation and haemagglutination assay:

Procedures were performed according to (OIE, 2008) by inoculation of tracheal sample homogenate into SPF embryonated chicken eggs. The tracheal tissue homogenates of suspected AIV subtype H9 and IBV were only used for egg inoculation. The collected allantoic fluids of suspected AI H9 subtype were tested for haemagglutination by slide haemagglutination test using 1% chicken red blood cells.

3. RESULTS

3.1. RT-PCR

To assess the efficacy of PCR for detection of AIV and IBV, RT-PCR was performed using the following positive controls: IB live vaccine, H5, H9 isolates RNA. The resulting PCR products obtained from the positive controls were of the expected sizes (Fig. 1 A, B, and C). On samples

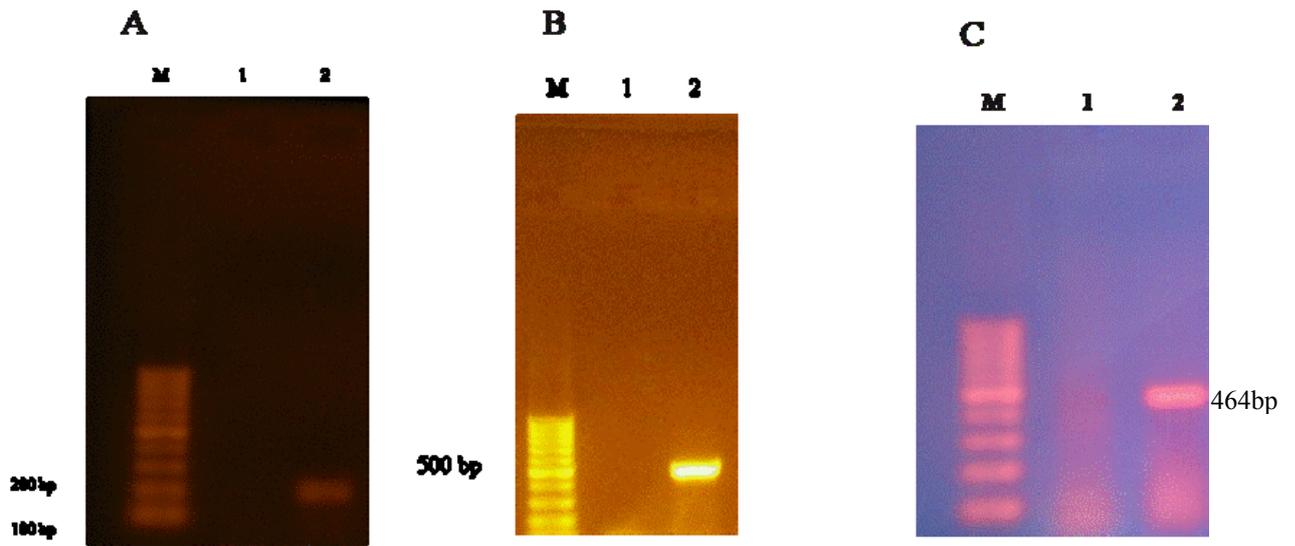
examination, out of 18 flocks, 6 flocks (33.3%) were positive for AI subtype H9, two flocks (11.1%) were positive for AI subtype H5, two flocks (11.1%) were positive for IBV, two flocks (11.1%) were positive for both AI subtype H9 and IBV, and 6 flocks (33.3%) were negative for all of these viruses (table 1).

3.2. Egg inoculation and haemagglutination assay

H9 positive samples haemagglutinated chicken RBCs following the second passage in SPF eggs. Most of the IBVs were isolated by two or three passages using 10-day-old embryonated chicken eggs.

The presence of IBV in tracheal homogenates (inoculums) was initially determined by IBV-specific RT-PCR as described previously and further confirmation was obtained by observation of characteristic embryonic changes such as dwarfing, stunting or curling of inoculated embryos (Fig. 2).

Fig. 1 RT-PCR of the positive controls



RT-PCR products of positive controls (a) M. DNA ladder 100 bp Lane 1, negative control, lane 2, PCR product of AIV subtype H5N2 (189) (b) M. DNA Ladder 100 bp, Lane 1, negative control, lane 2 PCR product of AIV subtype H9N2 (488 bp) (c) M. DNA ladder 100 bp, Lane 1, negative control, lane 2, PCR product of IBV attenuated vaccine (464 bp)

Fig. 2 Comparison of normal embryo (left) and curled, dwarfed IBV infected embryo of the same age (right). SPF eggs were inoculated with tracheal tissue homogenate from IBV infected flocks.



Table 2: One step RT-PCR method for detection of IBV and AIV H5 and H9 subtypes in tracheas from 18 chicken flocks

Flock number	IBV	H5 subtype	H9 subtype
1	+	-	-
2	+	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	+	-	+
8	-	-	+
9	-	-	-
10	-	-	+
11	+	-	+
12	-	+	-
13	-	-	+
14	-	-	-
15	-	-	+
16	-	-	+
17	-	-	+
18	-	+	-

4. DISCUSSION

In the present study, AIV subtypes H9 and H5 and IBV were investigated in the outbreak that affected commercial poultry flocks in Egypt during the winter of 2012. The involvement of AIV and IBV has been suspected due to some reasons. Firstly, Egypt is endemic with AIV subtype H5 N1 since 2006 (Abdelwhab and Hafez, 2011). Secondly, epidemiological observations and post mortem lesions of the affected birds suggested involvement of AIV and

IBV. The overall results of the current study showed that the percentage of detection of AIV in examined flocks was the highest (61%). The majority of AIV positive flocks were affected with H9 subtype. The presence of AIV H9 subtype in Egyptian poultry flocks is in agreement with the finding of (El-Zoghby et al., 2011). They isolated H9N2 virus from commercial bobwhite quail (*Colinus virginianus*), whereas the infected birds showed neither clinical signs nor mortality. They also suggested that the circulating H9 subtype in Egypt has been introduced from neighbouring countries rather than a separate introduction from Eastern Asian AIV. Their suggestion was based on close sequence and phylogenetic identities between Egyptian isolate and those of the nearby countries. Although, H9 subtype viruses do not satisfy the criteria for highly pathogenic avian influenza, one of the possible explanations for such a high mortality could be that it is due to mixed infection of the virus (H9 subtype) with other respiratory pathogens. This explanation could be supported with the reports of mortalities that occurred in the region such as Tunisia (Tombari et al., 2011), Jordan (Roussan et al., 2009), and Iran (Haghighat-Jahromi et al., 2008). For example, Influenza A viruses H9N2 have caused many outbreaks in Tunisian flocks in late 2009 and a second wave was reported during July–October 2010 (Tombari et al., 2011). Two flocks showed mixed infection between IBV and H9 subtype. The co-infection of IBV with H9 subtypes could be a factor in increased the severity of AI infection. The reason for that may be due to the cleavage activation of the HA. The cleavage of the HA play a key role in viral pathogenicity. Trypsin-like proteases are necessary for the cleavage activation of the AIV HA (Bosch et al., 1979, Klenk and Garten, 1994). It has been reported that a trypsin-like serine protease domain is encoded by coronavirus IBV (Liu et al., 1995, Ng and Liu, 2000). Therefore, IBV co-infection may have provided the

enzymes that enhanced H9 subtype pathogenicity. It was found that co-infection of IBV and H9 subtype could extend the period of AIV viral shedding that may affect clinical signs, postmortem examination and embryo studies (Haghighat-Jahromi *et al.*, 2008). In addition, the presence of similar enzymes in the field situation could possibly increase the pathogenicity of AIV H9 subtypes. There is general agreement that clinical signs and disease effects of AI may be far more devastating in the presence of bacterial pathogens, environmental conditions and other stresses. For explanation of this phenomenon, other researchers have proposed various hypotheses. These hypotheses are: secretion of trypsin-like proteases by bacteria, stimulation of host cells to produce or secrete more protease, destruction of endogenous cell protease inhibitors and suppression of the immune system due to stress by bacterial infection (Haghighat-Jahromi *et al.*, 2008). IBV infected flocks without involvement of AIV could indicate the important role of IBV in induction of mortalities. However, the RT-PCR positive results should be further confirmed. Because RT-PCR assay alone cannot differentiate IBV field strain from vaccine strain. Nucleotide sequencing of S1 glycoprotein is the only method to discriminate between all IBV strains. IBV is a highly contagious pathogen. Mortality in young chicks is usually 25-30%, but in some outbreaks can be as high as 75%. IBV also could have immunosuppressive effect due to its effect on bursa of Fabricius (Murphy *et al.*, 1999). Thus, IBV would be an important factor in mixed infection. Therefore, its presence with other pathogens, e.g., *E.coli* and *Mycoplasma gallisepticum* will aggravate their adverse effect. Six flocks out of 18 flocks were negative for AIV and IB according RT-PCR results. The lack of detection of such viruses in these flocks excluded their role as the sole cause of mortalities and respiratory symptoms. Therefore, the cause of

mortality and respiratory symptoms could be due to involvement of other pathogen such as Newcastle disease virus, *Mycoplasma gallisepticum*, *Escherichia coli* or other immunosuppressive pathogens. It can be concluded that the influenza A viruses H9 and other respiratory pathogens such as influenza A viruses H5 and infectious bronchitis virus could have an important role in mortalities that have occurred in Egyptian commercial poultry flocks during the winter of 2011-2012. The current study could indicate that there is no a single pathogen is responsible for the outbreak that occurred recently in Egyptian poultry flocks. Also, our findings revealed that H9 virus infection is well established in some Egyptian endemic areas.

Therefore, further work is needed for understanding the genetic and the biological characteristics of H9 virus isolated from different flocks to provide a comprehensive insight into the biology of H9, the ecology of AI virus, the ability of migratory birds to disseminate influenza viruses, and the ability to transmit to humans.

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دور فيروس انفلونزا الطيور (H9 و H5) وفيروس التهاب الشعب الهوائية المعدي في نسب النفوق العالية التي أثرت على أسراب الدواجن التجارية في مصر خلال عام 2012

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الملخص العربي

يعد فيروس انفلونزا الطيور (AIV) و فيروس التهاب الشعب الهوائية المعدي (IBV) من أهم مسببات الأمراض الفيروسية في الدواجن. في الآونة الأخيرة، عانت قطعان الدواجن التجارية في مصر من نسب وفيات ضخمة مرتبطة أساساً بأعراض تنفسية. ومن ثم قد أشتبته في علاقة AIV و IBV بهذا النفوق. في هذه الدراسة، تم اختبار عينات القصبة الهوائية من 18 قطيع من الدجاج المصاب خلال الفترة من يناير إلى مارس 2012 لفيروسات (H5, H9) AIV و IBV باستخدام خطوة واحدة عكس النسخ PCR (RT-PCR). وقد تم تأكيد نتائج RT-PCR الإيجابية عن طريق حقن خليط الأنسجة التي تمت تصفيتها في البيض الخالي من مسببات الأمراض المحددة (SPF) يليه أداء التراص الدموي لفحص سائل allantoic. أظهرت نتائج RT-PCR أن 6 أسراب كانت إيجابية للـ AI النوع الفرعي H9، كان اثنان أسراب إيجابية للـ AI النوع الفرعي H5، كان اثنان أسراب إيجابية للـ IBV، وكانت 6 أسراب سلبية لكل هذه الفيروسات. وأظهرت بيانات الدراسة أن هذه الفيروسات يمكن أن يكون لها دور هام في الوفيات التي حدثت في قطعان الدواجن خلال فصل الشتاء من عام 2012. مزيد من الدراسات الاخرى لتقييم السلالات المنتشرة وتأثير العدوى المشارك لهذه الفيروسات مع مسببات الأمراض الأخرى يعد أمراً لازماً.

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