

## Diagnosis and histopathological study of avian influenza virus-H5 (AIV-H5) in broiler farms

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(Received April 29, 2019; Accepted June 24, 2019)

### Abstract

This study was conducted for diagnosis and description of the pathological changes of AIV-H5 as the causative pathogen in Iraqi broiler farms. The current study was carried out on 84 broiler farms. Infected birds were tested for detection of the AIV infection from the tracheal swabs by rapid chromatographic AIV type A and H5 test kits. In RRT-PCR 8 samples (8 farms) of Trachea were selected to be tested by this assay. Samples of trachea, lung, and spleen from the dead birds with natural AIV-H5 infection were submitted for histopathological examination. seventy-two out of 84 farms tested for AIV-Type A gave positive results, and 58 out of 72 positives for type A-AIV gave a positive result for H5 antigen in a rapid chromatographic strip. The main gross lesions in the trachea of infected birds were severe congestion and hemorrhage. In the RRT-PCR assay, 8 out of 8 samples gave a distinct positive result for this test. The microscopic histopathological examination of infected tracheas showed obvious desquamation of lining epithelium with complete loss of cilia associated with congestion of blood vessels in lamina propria. Infected lungs revealed diffuse alveolar damage and severe multifocal vascular congestion. There was deposition of fibrinous material in the splenic tissue associated with the disappearance of the germinal centers. Thus, we concluded that AIV-H5 infection causes severe pathological and histopathological changes as a result of systemic infection. The RRT-PCR assay was highly sensitive and specific for the detection of highly pathogenic avian influenza virus subtypes.

**Keywords:** Avian influenza, RRT-PCR, Histopathology, Rapid test, Broilers

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### دراسة تشخيصية ونسجية مرضية لفايروس إنفلونزا الطيور النمط - ٥ في حقول فروج اللحم

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### الخلاصة

لقد أجريت هذه الدراسة لتشخيص ووصف التغيرات المرضية المصاحبة للإصابة بفيروس إنفلونزا الطيور - النمط H5 كمسبب مرضي لحقول الفروج اللحم في العراق. شملت الدراسة ٨٤ حقل للفروج. تم اختبار الطيور المصابة للكشف عن الخمج بفيروس إنفلونزا الطيور في مسحات القصبة الهوائية عن طريق اختبار الاستشراب المناعي السريع (للنمط A و H5). تم إجراء اختبار تفاعل البلمرة المتسلسل ذي الوقت الحقيقي على ٨ عينات من القصبة الهوائية لثمانية من هذه الحقول، كما تم إجراء الفحص النسجي المرضي لعينات القصبة الهوائية والرئة والطحال من الطيور النافقة والمصابة طبيعياً بالنمط H5. أظهرت النتائج أن ٧٢ من ٨٤ حقل تم اختبارها لفحص الاستشراب المناعي السريع نوع A أعطت نتائج إيجابية و ٥٨ من ٧٢ حقل كان موجباً للنوع H5. وتميزت الأفات الرئيسية في القصبة الهوائية المصابة بالاحتقان الشديد والنزف. وفي اختبار تفاعل البلمرة المتسلسل ذي الوقت الحقيقي للكشف عن فيروس أنفلونزا الطيور نوع AIV - H5 تبين أن ثمانية من مجموع ثمانية عينات أظهرت نتائج إيجابية لهذا الاختبار. أظهر الفحص النسجي المجهر لعينات

القصبة المصابة وجود انسلاخ لظاهرة القصبة الهوائية مع فقدان كامل للأهداب واحتقان الأوعية الدموية. وفي الرئة المصابة لوحظ تحطم الأسناخ واحتقان متعدد البؤر حول الأوعية الدموية. كما كان هناك ترسب من المواد الفبرينية في أنسجة الطحال مع اختفاء المراكز الانتاشية. وبهذا تلخصت نتائج هذه الدراسة إلى أن الإصابة بفيروس إنفلونزا الطيور- النمط H5 سبب تغيرات مرضية ونسجية نتيجة للخمج الجهازي. وان الحساسية العالية والسرعة والنوعية التي يتميز بها اختبار تفاعل البلمرة المتسلسل ذي الوقت الحقيقي في الكشف عن الأنواع المصلية لفيروس أنفلونزا الطيور شديد الإمراضية.

## **Introduction**

The highly pathogenic avian influenza virus (AIV) subtype H5N1 is considered one of the important pathogens that lead to severe respiratory disease with high morbidity and mortality in poultry industry. The H5N1 virus has firstly recorded in republic of China in 1996 and currently causes infection in worldwide including Asian, African and European countries (1). Since 1996, H5N1 subtypes undergone significant antigenic drift phenomena and these viruses have been classified into different clades according to phylogenetic features depended on hemagglutinin (HA) gene (2). The rapid diagnostic kits for detection of H5 subtypes are necessary for the control of infection with influenza viruses and these kits are affordable in the market for detection of specific nucleoprotein (NP) of this virus. The colloidal test strip technique give a quick results and does not need sophisticated requirements or special expertness, This technique has many benefits such as simplicity, rapidity, low cost, direct results and no expensive materials and equipment's. Pathogenicity of AIVs varies considerably depending upon host species, infective doses and routes of infection (3). Virus cultivation on embryonated eggs is currently performed for tracheal and cloacal samples from avian spp. are considered as standard diagnostic procedure for isolation, detection and subtyping of influenza A viruses (4). Precise and quick diagnostic procedure for the detection of infection is very important, not only for controlling of disease, but also to facilitate early antiviral medications. Molecular diagnosis of AIV-H5 by RRT-PCR is a rapid choice. The results of subtyping of the causative virus may be available within 24 hours. A pair of primers has also recommended by World Health Organization (WHO) for detection of H5 subtype as a laboratory assay (5). Different of RNA viruses has been detected successfully by RRT-PCR assay with hydrolysis probes (6,7). RRT-PCR technique provides advantages of rapidity, viral loading analysis, sensitivity and specificity in comparison with standard protocol of RT-PCR.

This study aimed for identification of AIV-H5 as a causative agent for outbreaks of severe respiratory illness in Iraqi broiler farms during 2018 with description of main pathological changes during the course of the infection with this virus.

## **Martials and methods**

### **Broiler farms**

The study was carried out on 84 broiler farms. All birds in these farms were clinically suffered from respiratory signs, severe depression, nasal discharge, excessive secretions within the lumen of the trachea associated with severe hemorrhages and high mortality rates. A group of infected birds from each farm were tested for detection the AIV infection from the tracheal swabs by rapid chromatographic AIV test kits (type A) (Bionote, Korea) according to the manufacturers' instructions depend on a monoclonal antibody against the viral nucleoprotein (NP). Tested birds which gave Positive results with rapid chromatographic strip type A undergo for detection the AIV-H5 infection with specific rapid chromatographic test kits for H5 antigen (Bionote, Korea).

### **Tracheal gross Lesions**

Tracheas of infected and dead birds suffered clinically from respiratory signs were investigated for the gross lesions as they are the target organs for AIV-H5 virus.

### **RNA isolation**

The isolation of viral RNA from virus containing tissue (trachea) was carried out by using an SV Total RNA Isolation System according to the manufacturer instructions (Promega Corporation, Madison, Wis., catalog # Z3100).

### **Primers**

Two different sets of sequence specific primers were used targeting the hemagglutinin (HA) genes of the H5 subtype which include (8):

Forward primer

(5'-ACAAAGCTCTATCAAAACCCAAC-3')

Reverse primer

(5'-TACCCATACCAACCATCTACCAT-3')

### **Real-time Reverse Transcriptase/Polymerase Chain Reaction RRT-PCR**

Eight tracheal positive samples (for rapid chromatographic strip-H5) were randomly selected from infected birds to be used in this test. briefly the reactions were performed with the GoTaq<sup>®</sup>1- Step RT-qPCR Kit (Promega, USA, catalog # 6021) by using an applied biosystem step one Real-Time PCR system (48 wells, ABI,

USA). The temperature profile consist of an initial step  $\geq 37$  °C for 15 min for reverse transcription process, RT-PCR inactivation/hot-start activation step of 95 °C for 10 min followed by 40 cycles for 10 s at 95 °C for denaturation, 30s at 60 °C for annealing, 30s at 72 °C for elongation and a final extension step at 60-95 °C. The qPCR assay used applied Biosystems SYBR® Green universal PCR with master mix (25 $\mu$ l), 5  $\mu$ l of Forward primer, 5  $\mu$ l of reverse primer, 10  $\mu$ l of cDNA produced from reverse transcribed clinical sample, 5  $\mu$ l MgCl<sub>2</sub> and 5  $\mu$ l of CXR reference dye.

### Histopathology

Samples from the dead birds with natural AIV-H5 infection that detected by rapid chromatographic test were submitted for postmortem and histopathological examination. Tissue section of trachea, lung and spleen were removed and fixed in 10% of neutral buffered formalin. All tissue samples were embedded in paraffin wax, sectioned at 5  $\mu$ m, were stained on clean glass slides with Hematoxylin and Eosin (H&E) and Masson trichrome stains for histopathological examination by light microscope (9,10).

### Results

#### Rapid chromatographic strip

Seventy two out of 84 farms tested for AIV were positive (Table 1, Figure 1a) and 58 out of 72 positives for type A-AIV gave positive results for H5 antigen in rapid chromatographic strip (Figure 1b).

Table 1: Percentage and number of tested and infected farms with rapid chromatographic strip

|   |        |
|---|--------|
| Total number of farms tested for type A | 84     |
| Number of Positive AIV-A farms          | 72     |
| Percentage of Positive AIV-A farms      | 85.72% |
| Number of negative AIV-A farms          | 12     |
| Percentage of negative AIV-A farms      | 14.28% |
| Total number of farms tested for H5     | 72     |
| Number of Positive AIV-H5 farms         | 58     |
| Percentage of Positive AIV-H5 farms     | 80.55% |
| Number of negative AIV-H5 farms         | 14     |
| Percentage of negative AIV-H5 farms     | 19.45% |

#### Tracheal gross lesions

The main gross lesions in trachea of infected birds were severe congestion and hemorrhage in comparison with normal trachea (Figure 2).

#### Real-time Reverse Transcriptase/Polymerase Chain Reaction RRT-PCR

All positive samples with AIV-type A and then AIV-H5 were also positive for RRT-PCR in comparison with +ve and -ve control (Figure 3).

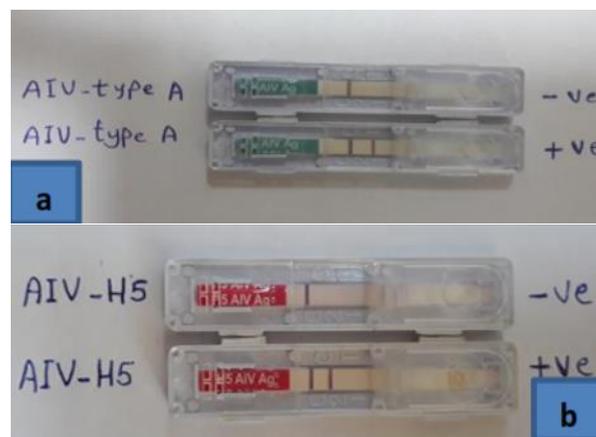


Figure 1: Positive and negative samples for AIV-A and H5 antigen using rapid chromatographic strip.

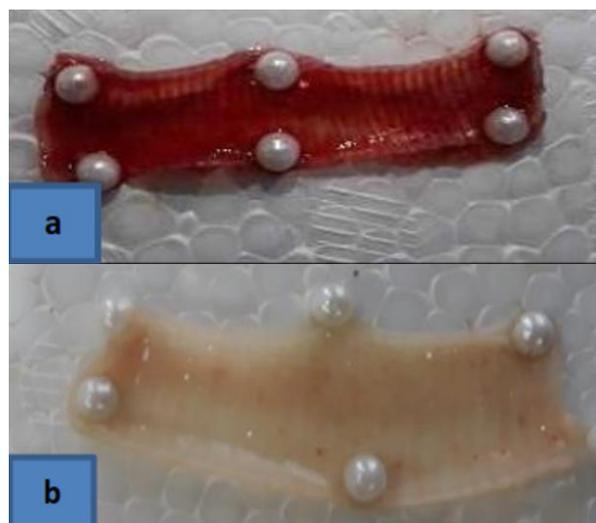


Figure 2: (a) Congestion and hemorrhage of infected trachea, (b) normal trachea.

#### Histopathological finding Trachea

The histopathological examination of infected trachea specimens showed obvious desquamation of lining epithelium with complete loss of cilia, extensive hemorrhage and infiltration of inflammatory cells especially macrophages, lymphocytes and plasma cells associated with congestion of blood vessels in lamina propria (Figure 4b) compared with control trachea in which the cilia are still intact (Figure 4a). Degeneration and necrosis of chondrocytes was evident in the hyaline cartilage (Figure 4c). Infiltration of inflammatory cells with coagulative necrosis and edema was prominent in the muscular layer (Figure 4d).

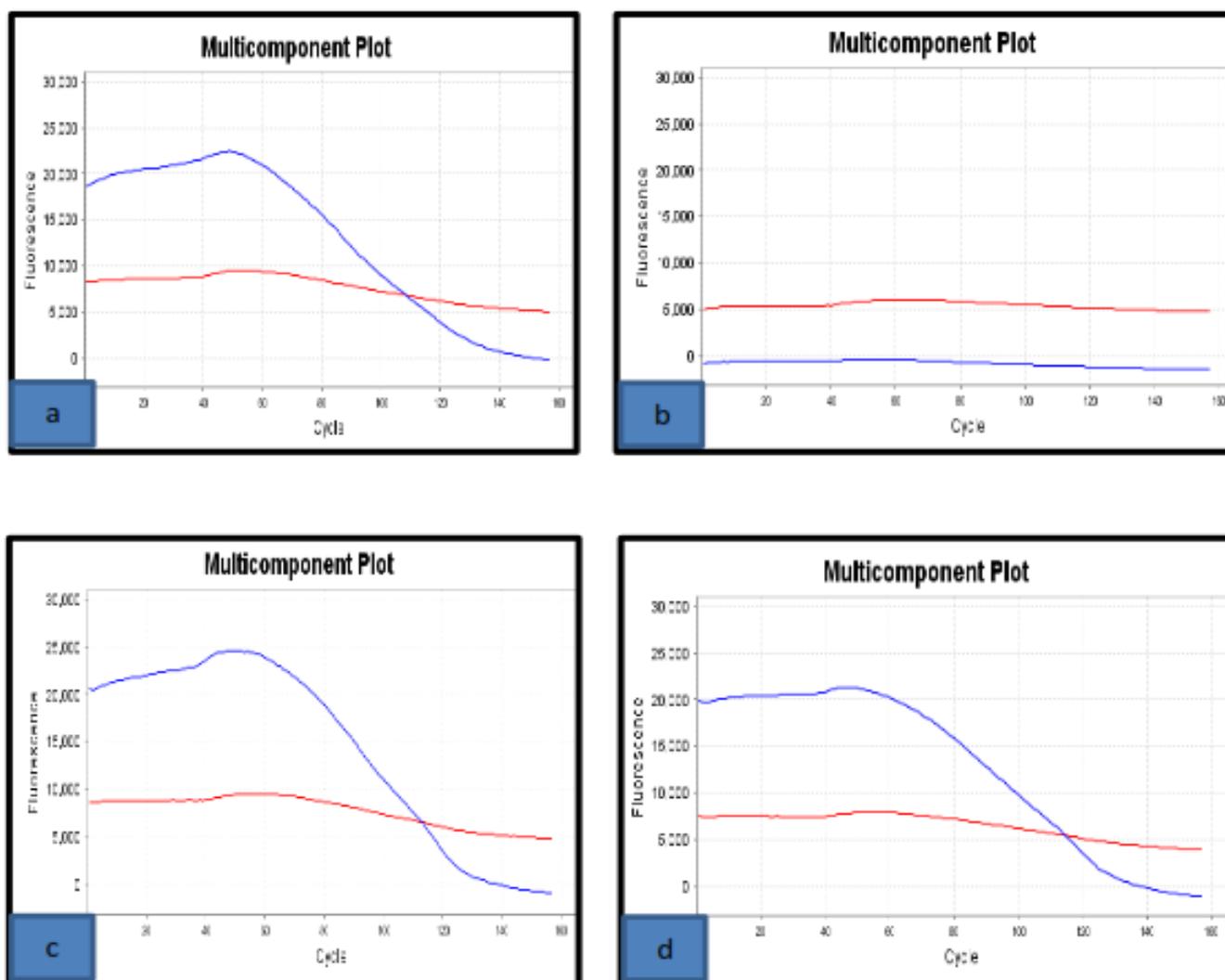


Figure 3: RRT-PCR assay examining cDNA from different samples infected with AIV-H5, a = +ve control, b = -ve control, c and d = 2 out of 8 +ve samples.

### Lung

Histopathological sections of infected lungs revealed diffuse alveolar damage and severe multifocal vascular congestion with extensive hemorrhage (Figure 5b). Interstitial fibrosis leads to thickening of interlobular septa, cystic dilatation of the air space and edema (Figure 5c). Interstitial fibrosis with infiltration of lymphocytes, macrophages and heterophils in the interstitial and alveolar lumen (Figure 5d). Normal section of lung (Figure 5a).

### Spleen

The histopathological changes of spleen from infected birds is characterized by multifocal necrosis of lymphoid cells with thickening of blood vessels wall lead to lumen stenosis (Figure 6a), was a deposition of fibrinous material in the splenic tissue associated with lymphocytes depletion and disappearance of the germinal centers (Figure 6b, c).

Figure 4: (a) Tracheal section of healthy broiler showing normal structure of trachea (blue arrow). (b) Tracheal section of infected broiler with AIV-H5 virus showing desquamation of lining epithelium with complete loss of cilia (green arrow) hemorrhage (red arrow) infiltration of inflammatory cells (organ arrow) and congestion of blood vessels. (c) Tracheal section of infected broiler with AIV-H5 virus showing degeneration (blue arrow). (d) Necrosis of chondrocytes of hyaline cartilage (blue arrow) coagulative necrosis and edema of muscular layer (yellow arrow). H&E, 10x.

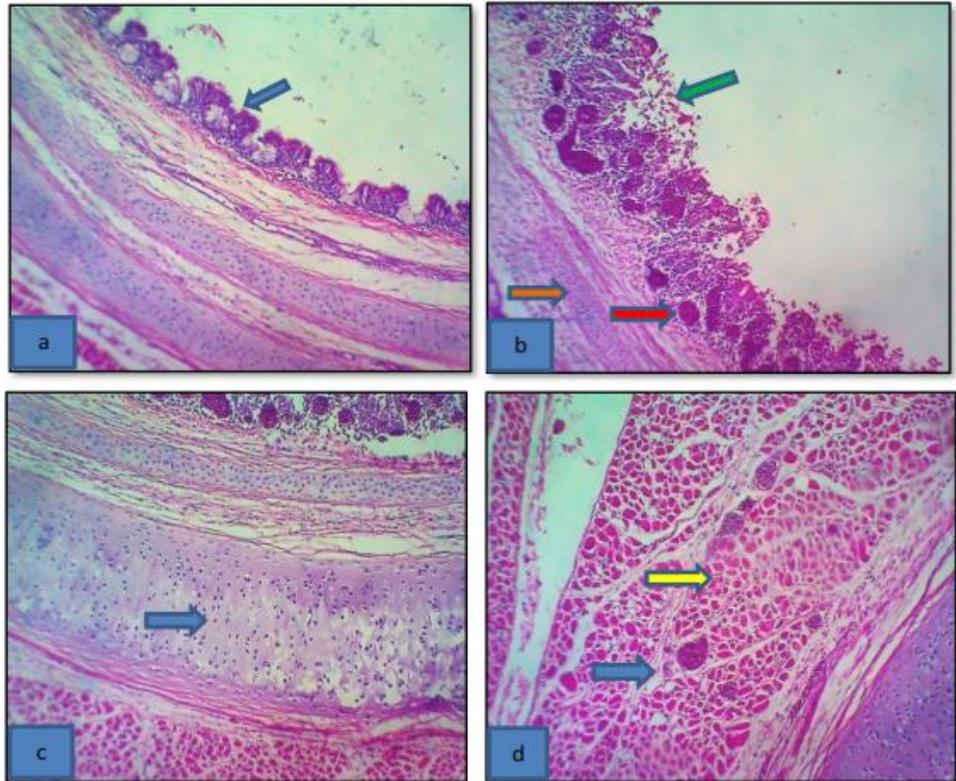
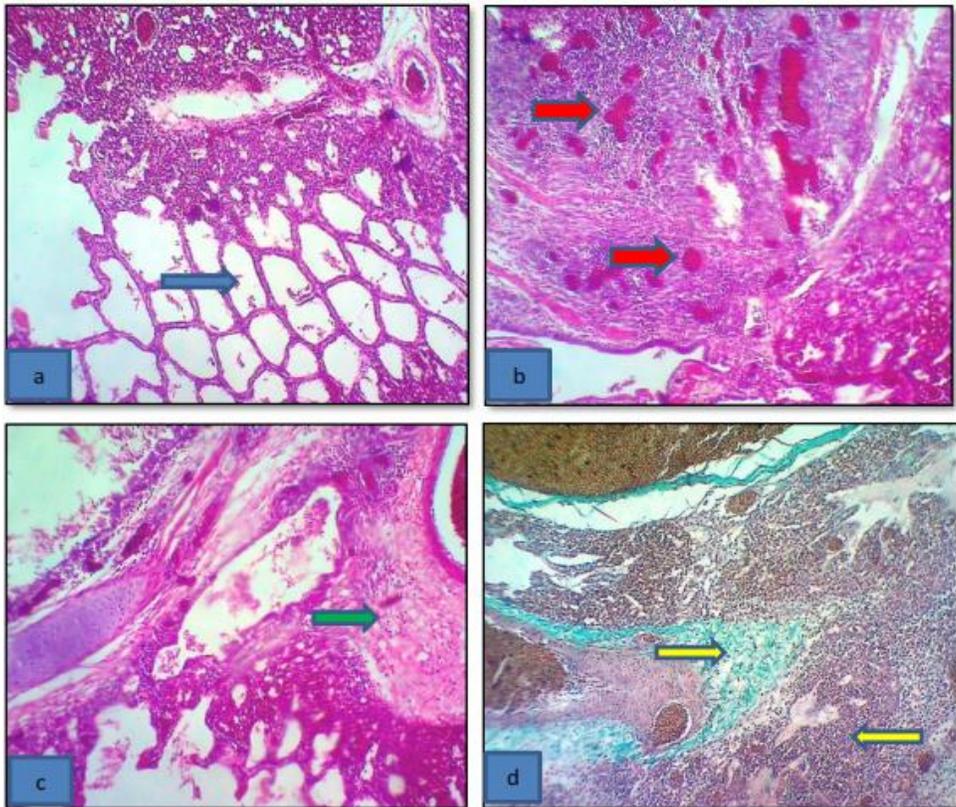


Figure 5: (a) Lung section of control broiler showing normal structure of lung (blue arrow). (b) Lung section of infected broiler showing vascular congestion (red arrow) and alveolar damage. (c) Section of infected broiler lung showing interstitial fibrosis (green arrow) and cystic dilatation of air space. (d) Infected section of broiler lung showing interstitial fibrosis with infiltration of inflammatory cells (yellow arrow) Masson's trichrome stain, H&E, 10x.



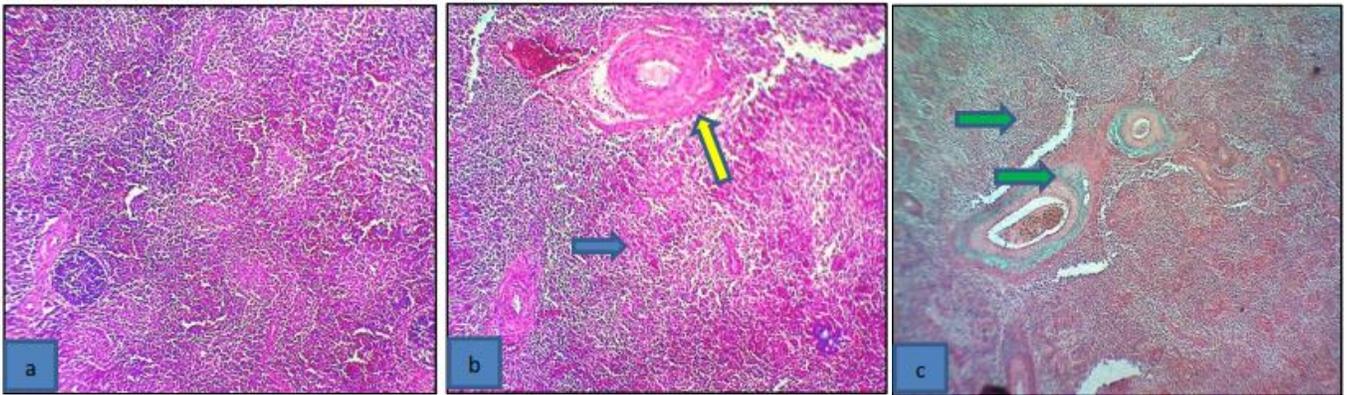


Figure 6: (a) Spleen section of control broiler showing normal architecture. (b) Spleen section of infected broiler showing focal area of necrosis of lymphoid cells (blue arrow) thickening of blood vessels wall and deposition of fibrinous material (yellow arrow), H&E, 10x. (c) Spleen section of infected broiler showing deposition of fibrinous material within splenic tissue and around the blood vessels walls (green arrow). Masson's trichrome stain, H&E, 4x.

## Discussion

During last three decades, there has been a noticeable increase in the occurring of highly pathogenic avian influenza virus (HPAI) outbreaks and in the numbers of avian species infected in those outbreaks (11). Viruses of subtypes H5 and H7 which classified as highly pathogenic avian influenza (HPAI) characteristically produce fatal and severe systemic infection with high mortality rate in poultry farms. The HPAI viruses replicates within the endothelial cells of target organs throughout the vascular system of chicken during primary infection (12). The pathogenesis of infection include invasion of virus to the blood-brain barrier which followed by infection of nervous system with concurrently attacking and replication in different parenchymal tissues and organs (13). The RRT-PCR assay has been used worldwide successfully for the detection of many pathogens including RNA viruses (5). Since the rapid diagnostic test kits which are used for the detection of the nucleoprotein of the virus do not identify the subtypes of influenza A virus. The percentage of infected farms with this type of test was 85.72%, However, in the present research, the rapid chromatographic kit was used to detect the HA spikes of H5 antigen gave a percentage of 80.55% in tested farms, this study agreed with Chua *et al* (14) who mentioned ranges from 34.4% to 81.3%. Moreover, the rapid feature of these diagnostic kits could accomplish within 10-15 min and need no specific indicators, with high sensitivity and specificity and its significance for urgent control measures of H5 HPAI viruses (15). In microscopical examination the severe hemorrhage of trachea agreed with Kim *et al* (16) and the congestion occur as a result of excessing the capillary vessels with infiltration of leukocytes in the lamina propria (17). In comparison to the previous study Spackman *et al* (18), the methods which

are used in the present study provide many benefits over classical diagnostic methods, including rapid, easy, accurate and flexible. The RRT-PCR assay provides several benefits than standard assay, since it is rapid test for detection of the AIVs subtypes, including identification of co-circulating viruses (19). The one-step RRT-PCR assay is sensitive and it did not need to isolate and cultivate of pathogens from clinical samples. In conclusion the RRT-PCR assay described here provides a quick, sensitive and specific method to detect the H5 gene of AIVs in the clinical samples of diseased poultry (20). The pathological changes include anatomical and histological changes which occur as a result of viral invasion and replication within the target cells of susceptible host tissue or organ (21). The replication of AIV-H5 within the respiratory epithelial cells leads to development and occurrence of histopathological changes of trachea and lung. In the previous study which showed that the AIV carried by vascular endothelial cells of infected birds lead to alteration in the permeability of blood vessels resulting in hemorrhage, infiltration of inflammatory cells and necrotic lesions in lung, trachea and spleen (22,23).

## Conclusion

In conclusion AIV-H5 infection cause severe pathological and histopathological changes as a result of systemic infection, the rapid chromatographic strips showed high specificity and sensitivity as field diagnostic tool. The RRT-PCR assay was highly sensitive and specific for detection of avian influenza virus subtypes with highly pathogenic properties (HPAI).

## Acknowledgement

Authors would like to thanks the College of Veterinary medicine .University of Mosul / Iraq.

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