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Identification and isolation of low pathogenic avian influenza (LPAI) virus from chicken farms in Iraq

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***Abstract*---**The objective of the study was Investigation and isolation of the Low Pathogenic Avian Influenza virus in poultry farms (Broilers and layers) in different provinces in Iraq over the period from August 2020 to June 2021. A total of 241 samples, including (Larynx, trachea & lunge) were collected from 31 poultry farms suspected to be infected with avian influenza and distributed in different provinces including Baghdad, Babylon, Wasit, Karbala & Salahuddin provinces. Collected samples were prepared firstly then viral RNA was extracted by a special viral RNA extraction kit and amplified by using primers for M gene-specific for type A influenza virus and primers specific for H9 gene of LPAI in Real-time RT-PCR which showed that from (31) farms suspected to have low pathogenic avian influenza, six farms (19.35%) were positive H9 LPAI. After that the positive H9 samples were isolated in chicken embryos, after processing these samples were inoculated into the allantoic cavity of (10) days old embryonated eggs with cultivation for four days, then the allantoic fluid (AF) was collected and stored in the deep freeze and the pathological lesions in the embryos were recorded, then the harvested (AF) was prepared and tested by Real-time RT-PCR to confirm presence of H9 LPAI.

***Keywords*---**low pathogenic avian influenza, real-time RT-PCR, isolation.

Introduction

Avian influenza (AI) is considered a dangerous viral disease that infected numerous types of birds and it is a highly contagious disease caused by different

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subtypes of Avian influenza virus type A from the Orthomyxoviridae family. AIVs is a segmented, negative-sense, single-strand RNA virus (Swayne *et al.*, 2008; Capua & Alexander, 2009), divided into subtypes based on two viral surface proteins, hemagglutinin (HA) and neuraminidase (NA) (Olson *et al.*, 2014). It's important veterinary and human health pathogens around the world, has been recognized since the late 19th century, caused several outbreaks and severe fatal diseases among humans, poultry, and a wide variety of birds and mammals (Swayne *et al.*, 2013; Suarez, 2017), and pose serious burdens and threats to global public health, resulting in annual economic losses from a global influenza pandemic are estimated to be about 500 billion US dollars per year and a huge economic losses for poultry industry (Suarez, 2010; Imamura & Oshitani, 2021). Influenza A viruses (IAVs) have a wide host range involving various wild and domesticated mammalian hosts within diverse environments, including humans, pigs, horses, dogs, bats, and sporadic infections in miscellaneous mammalian hosts. However, waterbirds of the orders Charadriiformes, shorebird species which include gulls, and Anseriformes which include geese, swan species, and particularly ducks are considered to be the primary natural reservoir of the virus (harbor 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes, which can combine to yield many varying subtypes or strains) (Osterhaus *et al.*, 2008; Suarez, 2010, 2017; Spackman, 2020; Wille & Holmes, 2020) which are preferentially infected by LPAI viruses and normally do not show any clinical signs. LPAI viruses replicate in the cells lining the intestinal tract of aquatic wild birds, are excreted in feces, and spread mainly via virus-contaminated water and fomites (fecal–contaminated–water–oral route) thus migratory wild waterfowl can spread the virus around the world (Kelly *et al.*, 2008; Sriwilaijaroen & Suzuki, 2014). The AIVs genome is composed of eight segments of single-stranded RNA with RNA-dependent RNA polymerase lacking proofreading mechanisms, therefore it is exposed to a high mutation rate (Monne *et al.*, 2014; Shors, 2017; Kim *et al.*, 2018; Naeem *et al.*, 2020). From what was mentioned above, AIVs have been difficult to control and still poses a significant threat to poultry flocks. The core of disease prevention and control relies on strengthened surveillance activities (Althaqafi *et al.*, 2021). Therefore, the present study provides an insight into the LPAI viruses in Iraq.

Material and Methods

Samples collection

Between August 2020 and June 2021, samples were collected from 31 chicken farms (Broilers and layers) suspected to be infected with avian influenza in four provinces representing center and east parts of Iraq (Babylon, Wasit & Karbala) distributed according to the following regions as shown in table (1). From different vaccinated and non-vaccinated farms with different ages and numbers of birds, suspected infected chickens were subjected to necropsy and, tissue samples were collected including larynx, trachea and lung (pooled together) per flock were collected in a sterile phosphate buffered saline in labeled containers and transported immediately with ice to the laboratory and stored at - 80°C until processing and virus detected by RT-PCR technique.

Table 1. number of samples, number of farm and regions of samples

No	Number of samples	Number of farms	Regions
1	85	17	Babylon
2	36	9	Wasit
3	24	5	Karbala
Total	145	31	

Viral RNA extraction

Samples first prepared in class 2 biological safety hood, the identification number of the related sample was written on each tube. Using sterile scissors, small blocks of tissues was cut from the organ under examination, and minced with help of sterile sand. Phosphate Buffer Saline (PBS) with antibiotics (Streptomycin, Crystalline penicillin at 1 mg/ml, 10,000 U/ml concentration respectively) in a 1:1 proportion was added. The homogenate was collected in a tube and centrifuged in a cooling centrifuge at 3000 rpm for 15 min at 4 °C and the supernatant was collected for RNA extraction. The viral RNA was extracted from the supernatant of precipitated grinded tissue samples according to instructions of the QIAamp viral RNA kit of QIAgen Germany company. Viral RNA was transferred in a clean 2 ml screw-cap tube and stored at -70 ºC.

Viral Nucleic acid amplification by real-time RT-PCR

Real-time RT-PCR was performed in a one-step protocol using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase kit of the USA Invitrogen company and according to instructions of the kit at which: In a specific clean cabinet, reaction mix was prepared by mixing 12.5 µl of 2X reaction mix, 1 µl of enzyme mix, 1 µl of each forward and reverse primers (table 2), 0.5 µl of fluorescent probe, 3.95 µl of DEPC water and 0.05 µl of ROX passive reference dye. The reaction mix for each detector was prepared in a separate tube. After preparing each detector mix, the reaction mix was dispended into a specific reaction plate according to previously prepared layout. The reaction mix was dispended at 20 µl /well, and the reaction plate was kept on ice during the whole process. After that, RNA extract was added to their specific wells per layout at 5 µl to get a final volume reaction of 25 µl/ well. The plate was sealed with specific thermal adhesive sheet, briefly centrifuged, and inserted in a pre-programmed thermal cycler to start the amplification process. The thermocycler was programmed at 1 hold of 50 ˚C for 5 minutes, denaturation step of 95 ˚C for 2 minutes followed by 45 cycles of 95 ˚C for 3 seconds as denaturation and 60 ˚C for 30 seconds as annealing and extension step. The thermocycler was programmed to measure fluorescence signal of FAM for M gene, and fluorescence signal of VIC from H9 at the end of annealing-extension step of each reaction cycle. After the end of the run, data were analysed according to the cycler operating software and results were recorded.

Table 2. Primers & fluorescent probe used in real-time RT-PCR

No	Oligo Name	Sequence (5'-3')
1	M+25	AGA TGA GTC TTC TAA CCG AGG TCG
2	M-124	TGC AAA AAC ATC TTC AAG TCT CTG
3	M+64Pr	FAM- TCA GGC CCC CTC AAA GCC GA- TAMRA (Spackman *et al.*, 2002)
4	H9F	ATG GGG TTT GCT GCC
5	H9R	TTA TAT ACA AAT GTT GCA YCT G
6	H9Pr	FAM - TTC TGG GCC ATG TCC AAT GG – BHQ1 (Hoffmann *et al.*, 2016)

Virus isolation

Virus isolation was performed according to the protocol adopted by (OIE, 2021). Samples first prepared, then the air space of (10) days old embryonated eggs was marked off and a suitable site of inoculation was picked on the egg shell and sterilized with ethanol 70% then 0.2 ml of the supernatant was inoculated into the allantoic cavity by using insulin syringe via air space of four (10) days-old embryonated chicken eggs and negative control inoculated with 0.2ml PBS only, the puncture hole in the egg was sealed by melted paraffin and incubated at 37°C with daily checking by candling for viability. Deaths on the first 24hr post inoculation (PI) were considered nonspecific death and neglected, while deaths recorded after more than 24hr to 96 hr post inoculation. The allantoic fluid (AF) from dead and surviving embryos were harvested after four hours chilling at 4 C in refrigerator with a sterile syringe and centrifuged at 3000rpm for 3 minutes to remove mixed blood and tissues debris and stored in sterile screw-capped vials at (- 80°C) till further use.

Results

Results of clinical examination

All samples were collected from fields suspected to be infected with avian influenza, showing variable clinical signs include mild to severe respiratory signs such as coughing, sneezing and rales, with gross lesions include hyperemia of tracheal mucosa and pulmonary congestion, in layers hens exhibit decreased egg production and eggs may be misshapen with loss of pigmentation of brown eggs. In addition to general clinical signs including huddling, ruffled feathers. lethargy, decreased feed and water consumption as show in figure 1-3.

Figure 1. A. depression & excessive ocular discharge (lacrimation) B. respiratory distress

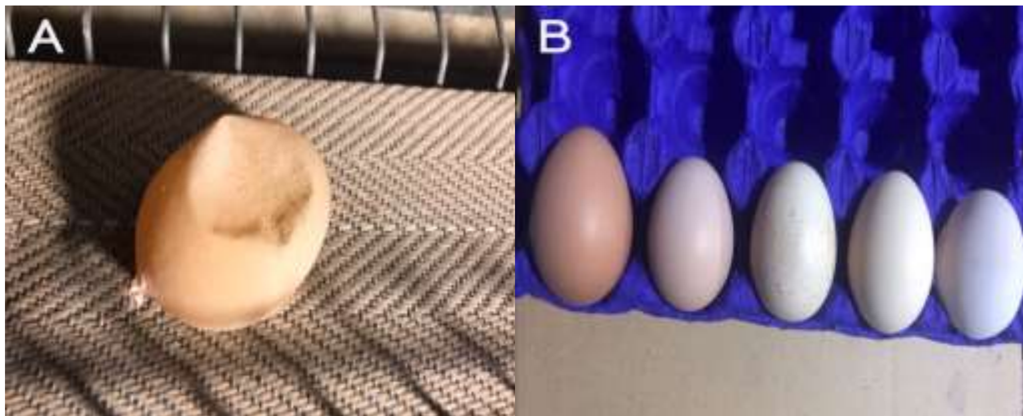

Figure 2. (A) shell-less eggs (B) loss of pigmentation of brown eggs

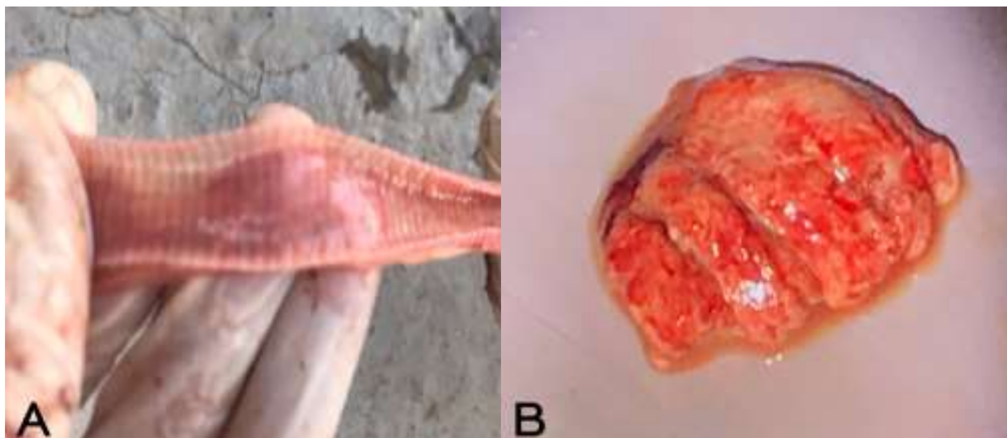

Figure 3. (A) congestion of the trachea. (B) pulmonary hemorrhage and edema

Real-time RT-PCR for M gene

The real-time RT- PCR was conducted directly after samples were prepared and nucleic acid extraction and the target was M gen to differentiate farms infected with AIV from others infected with other diseases. and the result showed seven farms outcome from thirty-one farms were infected with avian influenza and that equivalent 22.58%. The AI infection in broiler farms arrived to five farms 16.13% but the AI infection in layers was only two farm 6.45% (Table 3; Figure 4, 5 & 6).

Table 3. Samples Regions, M gene Ct. of avian influenza A virus using real-time RT-PCR & birds' type

Sample ID	Region	M gene Ct.	Birds type
1	Babylon / Al-Hilla	-ve	Layers
2	Babylon /Al Kifl	-ve	Broilers
3	Babylon / Al-Hilla	22	Layers
4	Babylon / Al-Hilla	-ve	Broilers
5	Karbala	-ve	Broilers
6	Karbala	-ve	Broilers
7	Babylon / Al-Hilla	-ve	Broilers
8	Babylon / Al-Hilla	24.3	Broilers
9	Karbala	-ve	Broilers
10	Babylon / Al-Mahaweel	21.2	Broilers
11	Babylon / Imam	-ve	Broilers
12	Wasit / Al Hayy	-ve	Broilers
13	Babylon / Al-Mahaweel	-ve	Layers
14	Babylon / Kothi	-ve	Broilers
15	Babylon / Musayyib	-ve	Broilers
16	Babylon / Musayyib	-ve	Broilers
17	Babylon / Kothi	-ve	Broilers
18	Babylon / Al-Hilla	-ve	Broilers
19	Babylon / Al-Hilla	-ve	Broilers
20	Babylon / Al-Hilla	-ve	Broilers
21	Babylon / Al-Hilla	-ve	Broilers
22	Wasit / Numaniyah	-ve	Broilers
23	Wasit / Numaniyah	-ve	Broilers
24	Wasit / Al Hayy	-ve	Broilers
25	Wasit / Numaniyah	30.3	Layers
26	Wasit / Al Hayy	25.8	Broilers
27	Karbala / Al- Hindiya	-ve	Broilers
28	Wasit / Kut	21.7	Broilers
29	Karbala	30.3	Broilers
30	Wasit / Numaniyah	-ve	Layers
31	Wasit / Numaniyah	-ve	Layers

Figure 4. Blot of real-time RT-PCR shows amplification specific for the M gene of avian influenza

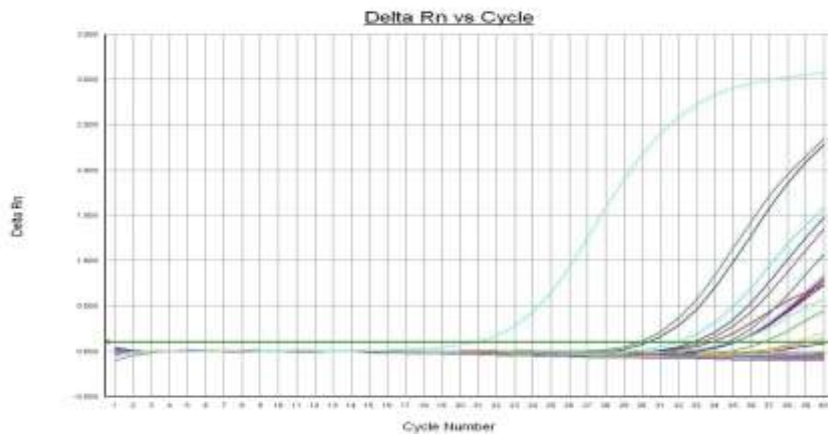

Figure 5. Blot of real-time RT-PCR shows amplification specific for the M gene of avian influenza

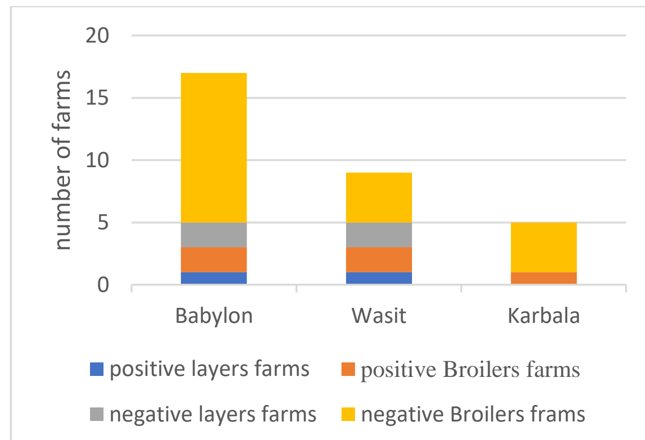

Figure 6. number of negative & positive farms (Broilers & layers) for the M gene in each province

Real-time RT-PCR of H9 gene

The molecular detection by using real-time RT-PCR was conducted on seven farms samples giving positive results with Real time RT-PCR for M gene to differentiated farms infected with LPAI from infected with HPAI by using a Primers specific for H9 gene, and the results showed six farms infected with LPAI (subtype H9). The results also revealed that the infections with LPAI in broilers were five farms which representing 16.12% of the total collected samples, and 83.33% of the total positive samples. While only one positive layer's farm which representing 3,22% of the total collected samples, and 16.67% of the total positive samples. table (4.) and figures (7) shows these results.

Table 4. positive LPAI samples, region, H9 Ct. and birds' type

NO.	Region	H9 Ct.	Birds type
1	Babylon / Al-Hilla	22.8	Layers
2	Babylon / Al-Hilla	25.2	Broilers
3	Babylon / AlMahaweel	21.5	Broilers
4	Wasit / Al Hayy	25.7	Broilers
5	Wasit / Kut	22	Broilers
6	Karbala	21	Broilers

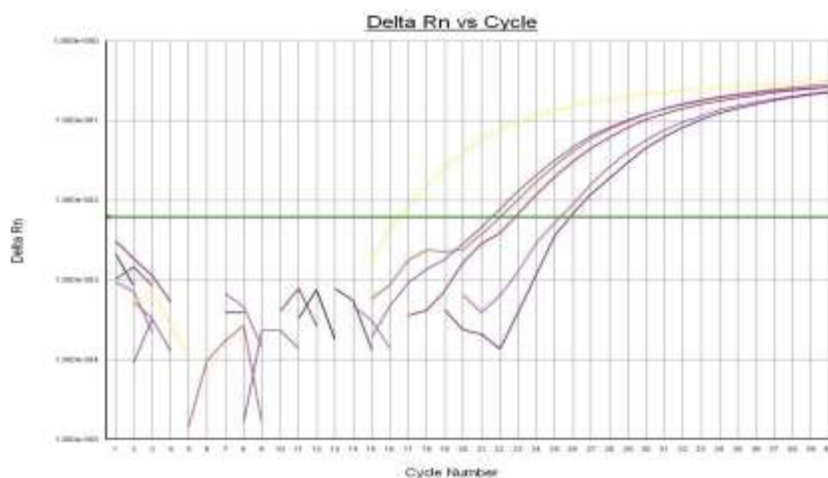

Figure 7. Blot of real-time PCR shows amplification specific for the H9 gene of avian influenza

Virus isolation by egg inoculation

After complete detection of H9 by using real-time RT-PCR and giving six positive samples, three of these six samples were isolated by using the intra allantoic cavity rote in embryonated chicken eggs. Each sample after processing with PBS then was inoculated into the allantoic cavity of four (10) days old embryonated eggs and incubated at (37°C) with a daily examination for viability. Death on the first (24hr) post inoculation (PI) were considered nonspecific death and neglected this egg, while recorded deaths after more than (24hr) to (96hr) post inoculation.

The allantoic fluid (AF) from dead embryos were harvested after four hours chilling at (4°C) in refrigerator and stored at (-80°C) till further use in real-time RT-PCR, and the results of isolation showed embryos with pathological lesion characterized by smaller size embryos than the control group embryos (traophied embryo), edema and diffusely red hemorrhagic head and body with three dead embryos as show in table (5) and figure (8 and 9).

Table 5 sample ID and dead embryos during the incubation period

NO.	Dead after 24h	Dead after 48h	Dead after 72h	Dead after 96h	Total Dead
1	-	-	1	1	2
2	-	-	-	1	1
3	-	-	-	-	-
Total	-	-	1	2	3

Figure 8. The left embryo shows the control negative virus inoculation (normal) and the right embryo shows the pathological effects of virus on the embryo after (96) hr. of virus inoculation

Figure 9. shows the pathological effects of virus on the embryo after (96) hr. of virus inoculation.

Real-time RT-PCR detection of isolated virus in chicken embryos

The stored harvested allantoic fluid (AF) of the three isolated samples were prepared and viral RNA was extracted and amplified by real-time RT-PCR using primers specific for the H9 gene to detect the H9 influenza virus and the H9 Ct value of the three isolated samples (1, 2, 3) respectively was (24.7, 25, 29) as in the figure (10).

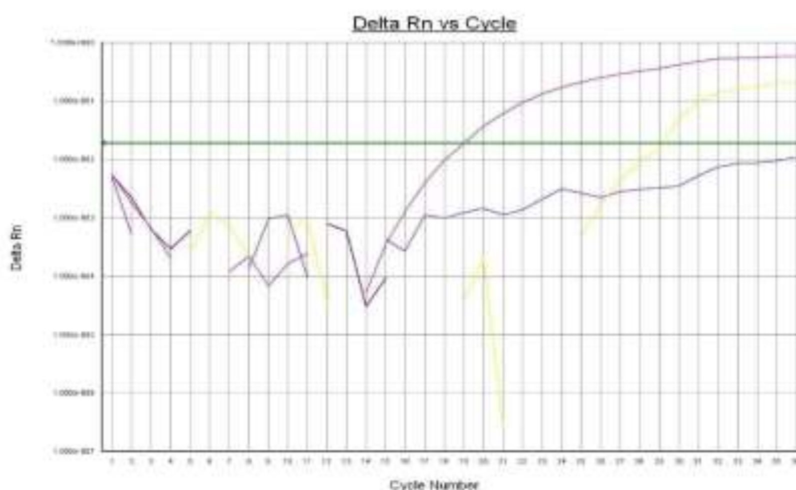

Figure 10. Blot of real-time RT-PCR shows amplification specific for the H9 gene of isolated virus

Discussion

The first step of the study was summarized by samples collection. LPAI is predominantly replicated initially the nasal cavity then release of virions and infection of other cells in the respiratory tract and the intestinal tract, illness or death is most often from respiratory damage (Swayne & Pantin-Jackwood, 2008), therefore (larynx, trachea & lung) samples were collected. All samples were collected from farms with case history of avian influenza infection. Clinical findings and post mortem examination were performed to chickens that displayed clinical symptoms with suspected avian influenza infection from different provinces and locations in center and east parts of Iraq as follows: Babylon 17 farms, Wasit 9 farms, and Karbala 5 farms. The clinical findings of birds include feed and water consumption decrease (Khamas, 2008) and birds in flock are notably quiet and depressed, ruffling feathers, mild-to-severe respiratory disease with signs of coughing, sneezing, rales, rattles, and excessive ocular discharge, and in layer flocks decreased egg production and misshapen eggs, figure (1 to 3) (Gonzales *et al.*, 2021; Gonzales & Elbers, 2018; Spickler *et al.*, 2008). Gross lesions are generally limited to hyperemia of tracheal mucosa and pulmonary congestion (Abdul-Aziz & Barnes, 2018; Pantin-Jackwood & Swayne, 2009). The next step was detection of the samples with real-time RT-PCR. The use of molecular-based methods (RT-PCR) for the detection of avian diseases in domestic and free-living birds has a great role in the early diagnosis and characterization of infections. The detection of RNA viruses as well as influenza viruses by real-time

PCR is high sensitivity, high specificity, rapid time-to-result (Spackman, 2020). samples were prepared and viral RNA was extracted by using special viral RNA extraction kit and run-in real-time RT-PCR targeting amplification of M gene to differentiated farms infected with AIV (type A influenza virus) from other farms infected with another diseases (Suzuki, 2005). and the result showed and the result showed seven farms 22.58% outcome from thirty-one farms were positives to the type A influenza M gene, then the positive seven samples were tested by real-time RT-PCR to detect the influenza viruses' subtypes in the samples and the results exhibited six positive (H9) LPAI subtype, and this result is in agreement with (Sabbar, 2007) who detect H9N2 LPAI in different regions of Iraq. The six positive samples represent only 19.35% of the total collected samples, because of the broad spectrum of signs and lesions reported with infections by AI viruses in poultry, LPAI virus infections may be confused with other diseases (Differential Diagnoses) such as NDV, infectious laryngotracheitis, infectious bronchitis, mycoplasma, and various bacteria (Kelly et al., 2008; Swayne et al., 2020). In spite of that, the percentage of this result also considered high in compared with (Hussein, 2019) which identified LPAI (H9N2) in Karbala province and show (6.3%) percent of infection because his study was restricted to the Karbala governorate only which limited the range of his search. The next step in this study was H9 positives samples isolation, LPAI virus isolation were began through inoculate the pro-prepared samples into the allantoic cavity of (10) days-old embryonated eggs via the air space according to the reference (OIE, 2021), and the embryos show macroscopic pathological changes characterized by congested head and body with dwarfing due to virus replication figure (8 and 9). This result agrees with (Abdul-Rassoul, 2008), And this method of isolation is considered the best method for AIV isolation and this method for isolation is used and confirmed by (El-Zoghby et al., 2012; Swayne et al., 2008).

Conclusions

Avian influenza virus was diagnosed and isolated from chicken tissue samples in several Iraqi governorates included in this study, which are: Wasit, Babylon and Karbala. The low pathogenic pathotype of subtype H9 were diagnosed in different broilers and layers farms. Influenza disease represents a real problem in the poultry industry in Iraq, which has caused great economic losses recently. The molecular-based methods real-time RT-PCR is a high sensitivity, high specificity, rapid time-to-result, and very helpful in the detection of avian influenza viruses.

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