International Journal of Psychosocial Rehabilitation, Vol. 24, Issue 10, 2020
ISSN: 1475-7192

Estimation the viral load and pathology of high pathogenic avian influenza virus (H_5N_8) in chickens

[1]AhmedNeamaMahmood, [2]Dr. Aida BaraAllawe

Abstract

Theaims of this study were detect the pathological changes and viral loadsofAIV subtype-H_5 as the causative pathogen in the industrial broilers farm in Iraq.In Iraq in the last 2017 and early 2018 and 2019 the high pathogenic avian influenza A subtype H_5N_8 virus was detected in several regions, and cause a high economic losses for the poultry industry.Avian influenza H_5N_8 is an infectious disease that mainly causes extreme respiratory illness in poultry that is associated with a high percentage of morbidity and mortality in the infected chickens. One hundred and fifty collected samples (trachea and lung) from different parts of broiler chicken from Baghdad were obtained during this study and neighboring governorates during the winter of 2019.The results of RT-PCR of collected samples showed that only 12 out of 150 tested samples were positive for H_5N_8.Real Time RT-PCR is consider asensitive, rapid, and practical molecular technique for detection of avian influenza disease viruses.Avian influenza viral loads were estimated in tissues from infected chickensby using quantitative real time RT-PCRtechnique to calculate the viral particles in each samples.The main clinical finding include depression, lack of agitate, ruffling feathers, and increase mortality of chicken, redness of wattle, hemorrhage of legs and subcutaneous tissues, swollen and edema of the face severe rals, sinusitis, edema of the head and wattle with diarrhea and even sudden death.Cross pathogenic lesion in post-mortem examination of chickenrevealedthat is accumulation of small quantities of yellowish color sero-fibrinous exudates and the walls of the thoracic air sacs are covered with fibrin and thickened with notable congestion of capillary blood vessels.congestion and petechial hemorrhage of internal organs(intestine & pancreas) with accumulation of sero-fibrinous exudate

Key works:- *Avian flu, high pathogenic avian influenza, H_5N_8.*

I. Introduction:-

Avian influenza (AI) is a disease caused by influenza type A virus from avian origin, these viruses cause a disease in domestic and wild avian and mammalian species, viruses of avian influenza are a single-

[1]*Department of Microbiology /College of Veterinary Medicine /University of Baghdad*
[2]*Department of Microbiology /College of Veterinary Medicine /University of Baghdad*

stranded with a negative sense RNA viruses bellowing to the family *Orthomyxoviridae*. Genome of avian influenza viruses consist of eight genomic segments[1].Based on two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), encoded by the genes (HA) and (NA), influenza A viruses have been classified. Internal gene caskets consist of six gene segments that code for the polymerase complex (basic polymerase 1 (PB1), basic polymerase 2 (PB2) and acid polymerase (PA), matrix proteins (M1 and M2), nucleoprotein (NP) and non-structural proteins 1&2 (NS1 and NS2). There are currently 18 subtypes of HA (from NS1 and NS2) [2].The enveloped RNA viruses are influenza viruses, and the family of Orthomyxoviridae currently consists of seven types: influenza A, influenza B, influenza C, influenza D, Thogotovirus and Isavirus. Long ago, the first four genera were recognized. A relatively new addition to *Orthomyxoviridae*, the Isavirus family contains an infectious salmon anemia virus, which is a debilitating pathogen in the world's fish farms. Influenza D is likely to be produced shortly because it is separated from cattle and swine[3].

Circulation of avian influenza viruses different hostsspecie may be lead to genomic exchange of their gene segments, which is subsequent leads to antigenic variation and creation of a new viral strains.This may cause serious outbreaks and epidemics, leading to tremendous economic losses for the poultry industry, and poses a serious threat to human and animals health [4].Hemagglutinin is the most common important gene ininfluenza virus replication and it plays a central role in influenza A viruses' life cycle through its participation in receptor recognition, virus attachment, membrane fusion and entry [5].

Hemagglutinin glycoprotein of Influenzavirion should be cleaved by a host proteases at a specific site to make a functional the receptor for entry to the target cells. The sequence at the hemagglutinin cleavage site determines whither easily it can be catalyze the host proteases enzymes. Hemagglutinin of low pathogenic avian influenza viruses can be cleaved by trans membrane serine proteases, and trypsin-like proteases in the airways, this limited cleavage ability restricts infection to certain tissues. Therefore, continuous surveillance of H_5 and H_7 subtypeof avian influenza viruses in is imperative to avoid distribution by this type of infection [7].

Avian influenza viruses strains with a high pathogenicity (H_5N_8) of the clade (2.3.4.4) were first detected among the wild birds in Asia during 2010, later it was spread to domestic birds across Japan, China, and South Korea, (8).More recently, a novel H_5N_8 subtype reassorting virus of the clade(2.3.4.4) was identified in Russia and spread to several countries in Asia, Europe and the Middle East(9).The spread of strains of H_5N_8 viruses has been related to the overlapping flyways of migratory wild birds coming from different continents(10) In Iraq the highly pathogenic strains of avian influenza viruses were reported in several governorates during the end of 2017 early 2018 and 2019, but the virus is not well characterized. In this study, the detection, viral load and histopathological changes of a H5N8 HPAI was described for isolates detected from domestic poultry.

II. Materials and methods:-

Samples collection and screening:-

During winter 2019, 150 samples including (trachea, larynx and lung) were collected from different areas from Baghdad and neighboring governorates, samples from suspected poultry farm which given symptoms of avian influenza illness. These farms showed a symptoms as ocular and nasal discharge, sneezing, coughing,

and enlarged infra-orbital sinuses. The mortality rate varies from 10% to 75%, subcutaneous and petechial hemorrhages on visceral organs and muscles in extreme inflammation, edema and red discoloration of the shanks and feet. Greenish diarrhea was common in badly affected birds , paralysis, and drooping of wings. These samples were collected in sterile container and cooled in ice bag and examined in the laboratory by RT-PCR test.

Samples preparation and RNA extraction:-

Samples were prepared and RNAwas extractedfrom collected samplesby using kit for total RNA extraction (KyltR RNA extraction Kit). After extraction RNA was elutedin 60µL of RNase-free DD water,(20 U) of RNase inhibitorwas added andRNA was stored at (-80 ºC) until using for Real-Time PCR.

Estimation of the H_5 AI viral load by qRT-PCR:-

After preparation of standard curve of six dilution tubes, the viral particles in each dilution were known, RNA from collected samples were introduced the reaction, the weight of each sample was taken by using a sensitive balance and the results of qRT-PCR were compared with the standard curve calculated previously, and the viral particles was calculated using specific software calculating system.

Histopathological examination of samples:-

Collected samples (trachea, larynx, lung , kidney and spleen) were examined for histopathological changes in comparison with healthy non infected and non-vaccinated chicken. These samples were fixed in buffered in 10% formalin for 24-48 hrs. after that the tissues were embedded in a low melting point of paraffin then sectioned at 5 ɱmthickness and then stained with Hematoxylin and eosin stains according to Luna, 1968[12].

III. Results:-

Clinical finding:-

All the samples were collected after clinical finding and post mortem examination of cases from chickens that give a clinical symptoms with suspected avian influenza infection from different provinces and locations of Iraq includes (Baghdad 75 samples, Dialla 42 samples, and Hilla 33 samples) from suspected poultry farm which given symptoms of avian influenza illness, these samples were collected based on information and case history, then the clinical signs and Post-Mortem examination.

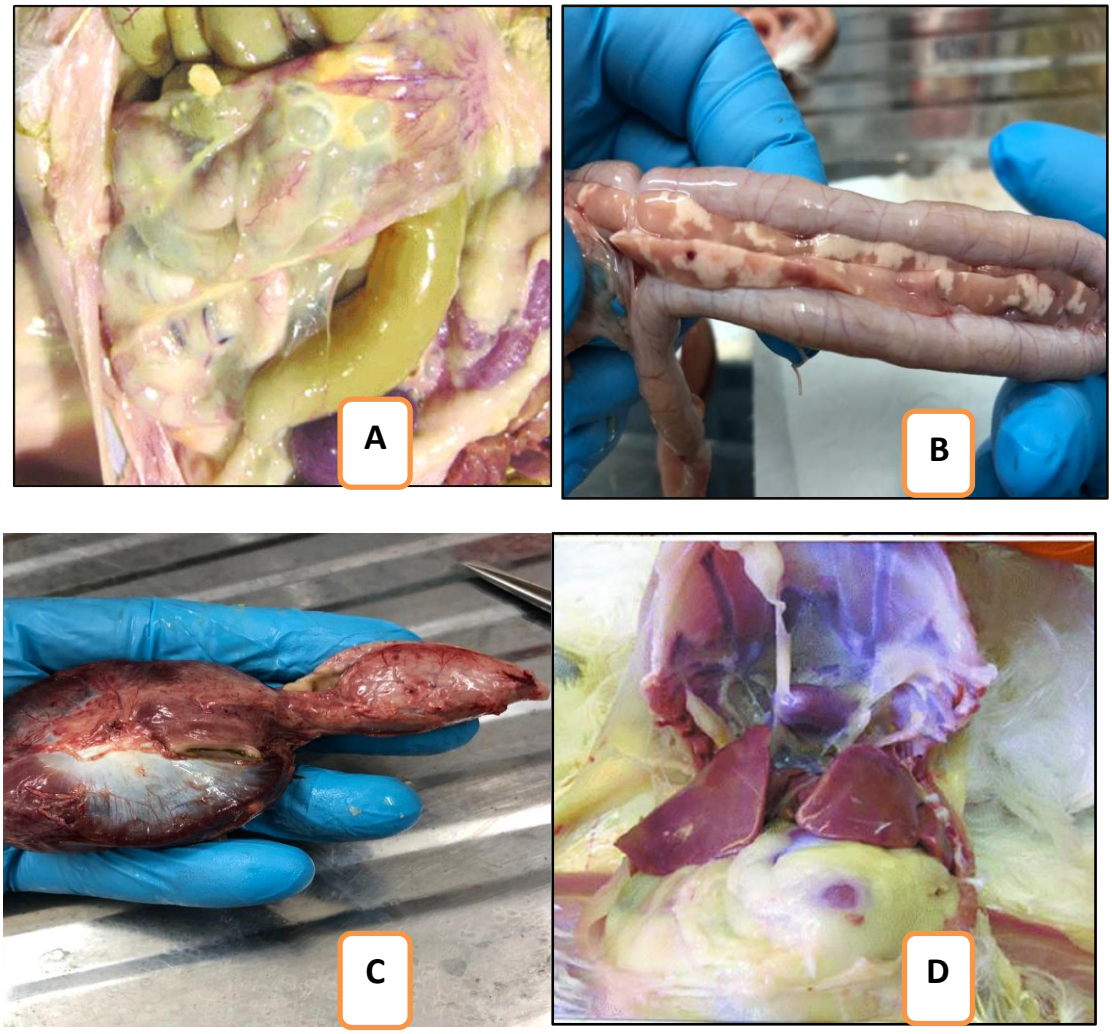
The main clinical signs were depression, lack of agitate, ruffling feathers, and increase mortality of chicken, redness of wattle, hemorrhage of legs and subcutaneous tissues, swollen and edema of the face severe rals, sinusitis, edema of the head and wattle with diarrhea and even sudden death.

Fig(1):- Clinical finding of chicken suspected to be infected with a high pathogenic avian influenza disease:-

A and B :- (clinical finding of chicken suspected to be infected with AI. Show sever edema with) red discoloration of feet andshanks due to subcutaneousecchymotic hemorrhages and congestion which is a pathognomic feature of avian influenza infections.

C :- (clinical lesion of chicken suspected to be infected with AI. Show sever petechial) hemorrhages on the thoracic muscles and other organs.

D :- (clinical lesions of chicken suspected to be infected with AI show cyanosis and edema of the head, comb and wattles).



Fig(2):- cross pathogenic lesion in post-mortem examination of chicken suspected to be infected by high pathogenic avian influenza disease:-

A) Small amountsof yellowish color sero-fibrinousexudates were accumulate and the walls of the thoracic air sacs are covered with fibrin and thickened with notable congestion of capillary blood vesicles.

B) Congestion and petechial hemorrhage of internal organs(intestine & pancreas) with accumulation of sero-fibrinous exudate .

C) There is sever congestion of blood vessels in the internal organs(Gizzard&Proventriculus) and diffuse petechial hemorrhage.

D) Thickened and cloudy walls of thoracic air sacs to gather with accumulation of sero-fibrinous exudates in the cavities, particularly in the posterior thoracic air sacs.

Results of qRT – PCR(viral load) of H_5 avian influenza:-

The result of serial dilution quantification of standard curve was performed according to the manufacturer instructions by using RT-PCR technique, this results were listed in the following table:-

Table (1)Results of stranded curve dilution series.

Si de ID	Result assay	Ct.	FAM con.	Sample type
A1	Standard	15.2	100 E07	STD1
A2	Standard	19	100 E06	STD2
A3	Standard	22.4	100 E05	STD3
A4	Standard	25.6	100 E04	STD4
A5	Standard	30.8	100 E03	STD5
A6	Standard	33.3	100 E02	STD6

Table(2) Results of quantification avian influenza viral particles in collected samples by using qReal-Time RT- PCR .

Sit ID	Sample ID	Canal result	Ct	No. of viral particles
A1	**1**	**PASS**	**24.1**	**929,460.58091±28 6307**
A2	**2**	**PASS**	**31.1**	**9,903.53697749±19614**
A3	**3**	**PASS**	**26.7**	**95,880.1498127±34082**
A4	**4**	**PASS**	**27.2**	**94,117.6470588±23529**
A5	**5**	**PASS**	**25.2**	**888,888.88888±8888**
A6	**6**	**PASS**	**26.3**	**11,711.02661596±581**
A7	**7**	**PASS**	**24.5**	**914,285.714285±71428**
A8	**8**	**PASS**	**27.4**	**93,430.656934±3065**

A9	**9**	**PASS**	**28.2**	**90,780.1418439±7163**
A10	**10**	**PASS**	**29.3**	**87,372.0136518±7713**
A11	**11**	**PASS**	**29.1**	**87,972.5085910±6529**
A12	**12**	**PASS**	**19.0**	**10.000000±6444**

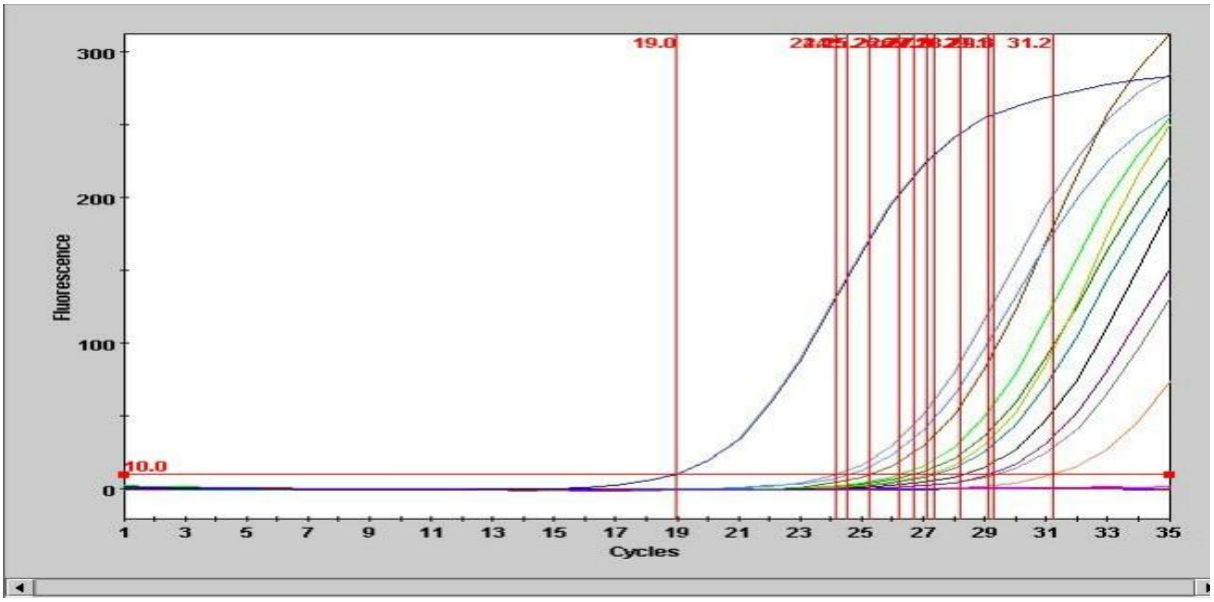
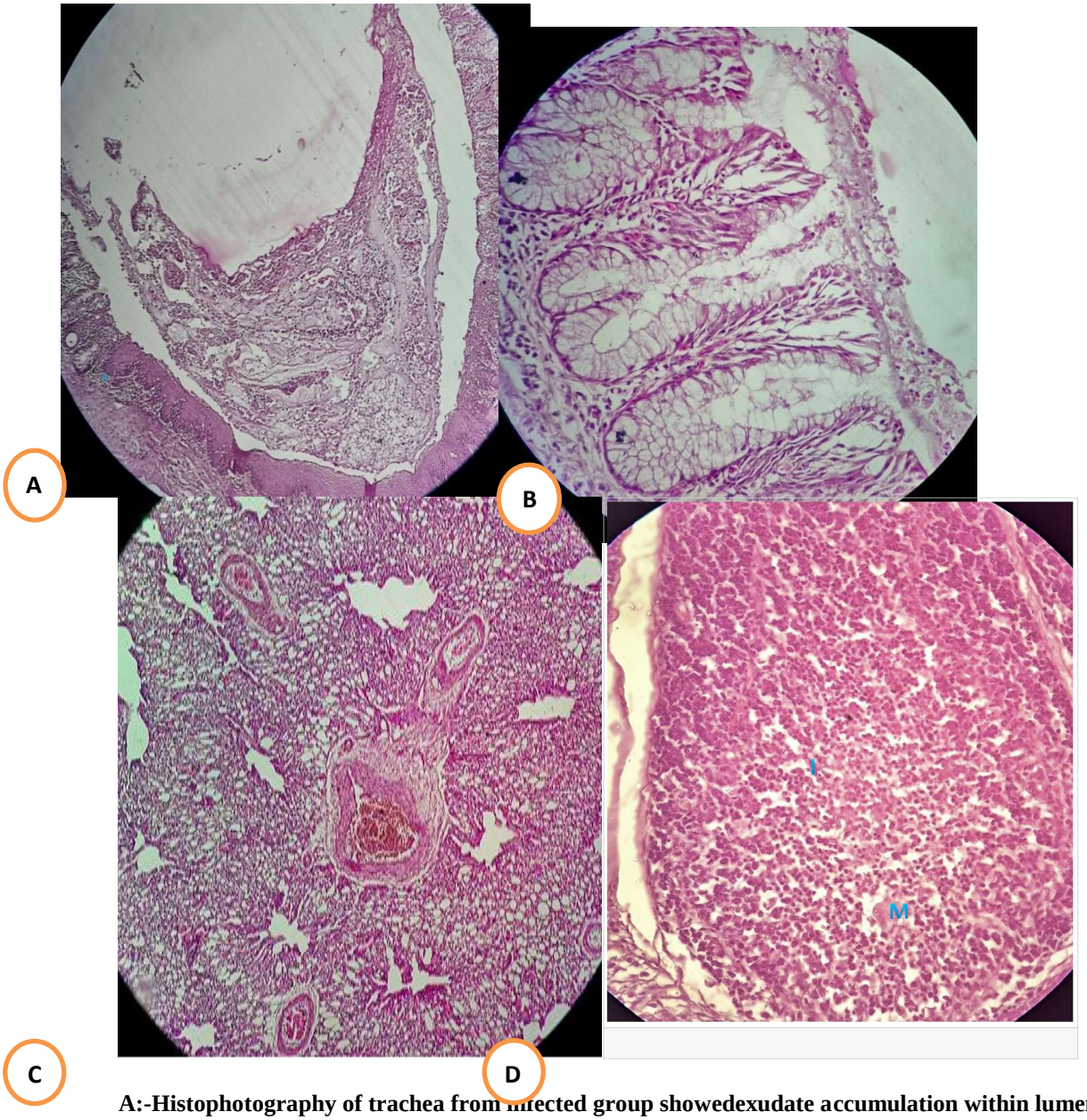


Fig (3):- Blot of amplification of H gene to quantification avian influenza viral particles(viral load) for H5 positive samples using qReal-Time RT- PCR .

Results of histo-pathological examination of samples:-

A positive samples of tracheas,kidney spleen and liver ofinfected chicken with HPAI subtype H_5 were examinedhistopathologically, The results of tracheas revealed there is desquamation of epithelial lining and marked aggregation of polymorphonuclear cells(PMN_C)and disappearance of cilia, in lungs tissues there is sever vesicular congestion with damage of alveolar tissue there is deposition of fibrineus material, in splenic tissues if infected birds and diffuse lymphoid depletion with manifestation of sinusoidal congestion.

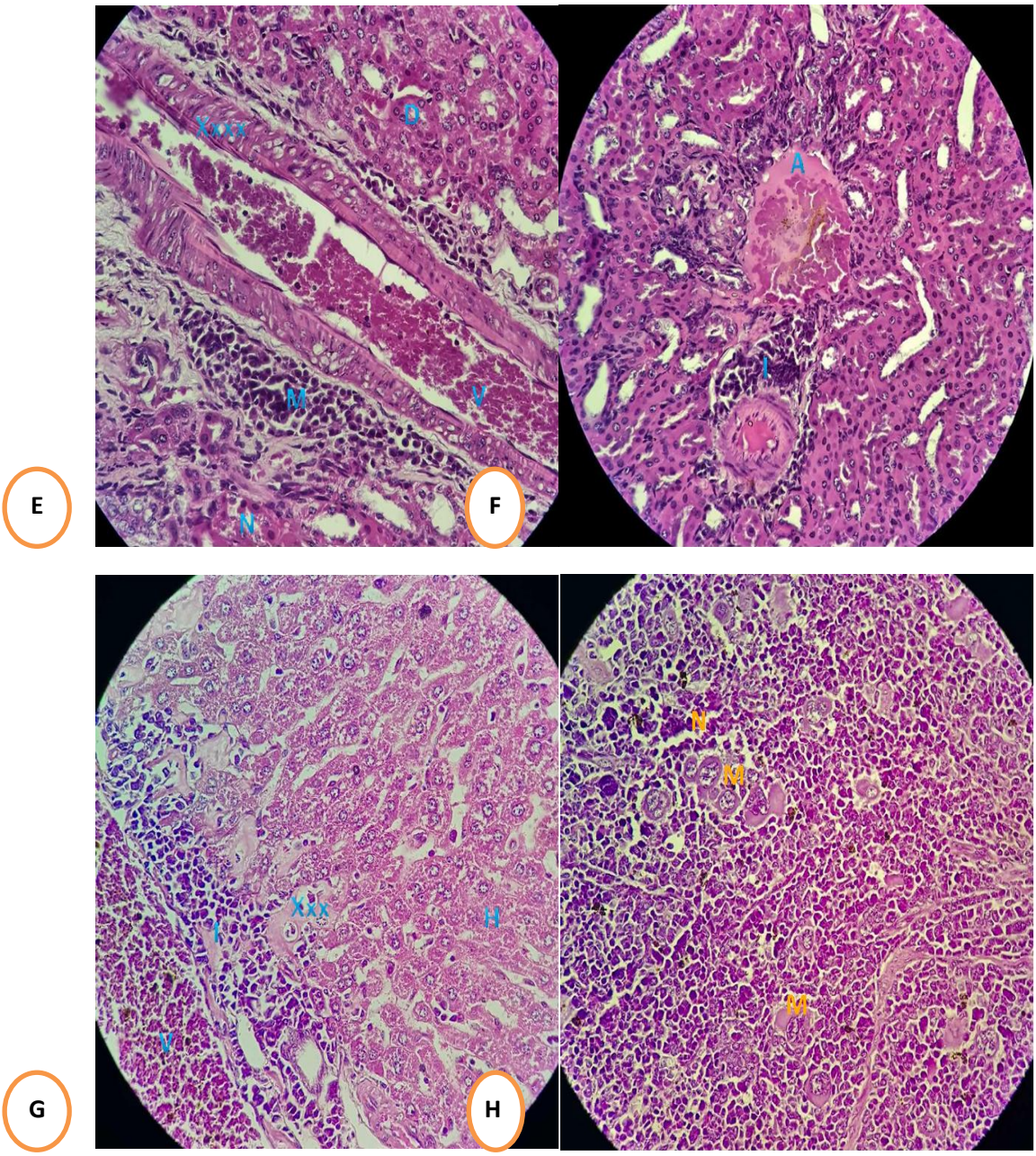


A:-Histophotography of trachea from infected group showedexudate accumulation within lumen and inflammation process in sub mucosa (H&E X10).

B:-Histophotography of trachea from infected group showed exudate accumulation within lumen(A) and goblet cell hyperplasia and hyper secretions (B) (H&E X10).

C:-Histophotography of lung from infected group showed dilated and congested blood vessels (H&E X4).

D:-Histophotography of larynx from infected group showed sever cell depletion (I) and megakaryocytes proliferation(M)(H&E X40).



E:-Histophotography of kidney from infected group showed congestion and dilated blood vessel (V), mononuclear cell infiltration (M), hydropic degeneration of epithelial cell (D) and coagulative necrosis(N) Endothelial cell hypertrophy(xxxx.)(H&E X40).

F:-Histophotography of kidney from infected group showed inflammatory cell accumulation (I) and amyloid like substance depositions (A) (H&E X40).

G:-Histophotography of liver from infected group showed hepatocellular necrosis (H), congestion of the blood vessel (V) and inflammatory cell aggregation (I) (H&E X10).

H:-Histophotography of spleen from infected group showed megakaryocytes hyperplasia (M) and sever focal necrosis (N) (H&E X40).

IV. Discussion:-

Avian influenza is a highly infectious extremely contagious respiratory viral disease,affected not only respiratory system but can affecting digestive, and/or nervous system of many species of birds of both domestic and wild birds, avian influenza is vary greatly in their ability to cause disease in birds from subclinical infection in wild birds to a severe illness with a high morbidity and mortality in case of a high pathogenic avian influenza infections (Hause, *et al.,* 2014). In the last few years there is markedly increase in the number of avian influenza outbreaks infections caused by subtype H_5& H_7 and also increase in avian species infections(Kapczynski., 2013).In Iraq, avian influenza with a high pathogenicity was reported in several governorates at the end of 2017, early 2018, and 2019, but the virus is not well characterized by a highly pathogenic avian influenza virus subtype H_5 that frequently causes human and animal disease and epically high economic losses in the poultry industry. The genetic characterization of H_5N_8 HPAI was identified in this study for isolates previously detected from domestic poultry and for viral load studies in domestic poultry.Real-time detection of RNA viruses and influenza viruses RT-PCR is considered a powerful, sensitive and practical molecular technique for the detection of AI disease viruses and has been used worldwide(WHO,2015). In this study AI subtype $H_5N_8$was detected by using H_5 and N_8 kit respectively from($Kylt^R$) company for veterinary diagnostic kits, the results of detection were in agreements with(Fanar*et al*.,2020) who was detected the presence of avian influenza subtype H_5N_8 by using real time PCR technique.Because of lack the continuously surveillance of avian influenza subtype H_5, therefore the HPAI viruses were still circulating in different country of the world (Lee *et al*., 2018).Clinical finding and macroscopical examination of infected birds as red discoloration of shanks and feet due to subcutaneous ecchymotic hemorrhages and congestion which are a characteristic lesion of influenza disease was agreed with(Kim et al., 2015). Histopathological and anatomical changes of infected tissues detected as a result of viral invasion, RNA replication and immune response against viral infection, desquamation of epithelial lining of trachea, focal deposition of pink hyaline like substances mixed with polymorphonuclear cells infiltration as a result of antibody-antigen reaction, in lung there is sever vesicular congestion with damage of alveolar tissue there is deposition of fibrineus material this results were agreed with (Swayne., 2007). Because the location of Iraq in the migration pathways between the black sea Mediterranean west Asian east African flay ways of the migratory birds it was suggested that H_5N_8 A/duck/Egypt/F446/2017 was originated from the same source and it was arrived to Iraq by means of migratory birds, this result was in agreements with(Kilpatrick *et al*.,2006) who suggest that the source if influenza infection was originated from the same avian source.Using RT-qPCRfor estimation of viral load in tissues is a useful mean to determine the number of viral particles that can produce infections, survival of viruses in the environments, shedding of viruses, pathogenesis of viral infection and evaluation of efficacy treatment. Viral load, also known as viral titer or viral burden, it is a numerical expression of the number of virus in a given volume of samples, these samples may be blood, plasma, tissues, or any other fluids(Wolfel*et al.,* 2020) The number of virus /ml can be determined by calculating the live quantity of virus in a fluid involved.In this study quantitative RT-PCR was used for the first time for detection of viral load in different organs and tissues infected by estimation of the RNA copies, in this technique, the number of virus /ml can be determined by calculating the live quantity of virus in a fluid involved, it is a useful means to determine the number of virus particles that can produce infections(I.D50), (EID50) and (LD50), as well as survival of viruses in the environments, shedding of viruses, pathogenesis of viral infection and evaluation of treatment (Puren, *et al.* 2010).AI viral antigens were mainly

localized in the endothelial cells of these systemic organs in accordance with the histopathological photo, which may affect the integrity of the blood vessels and cause bleeding in these organs. Therefore, in view of these observations were in agreement lesion of (Brojer*et al.*2009 and van Riel et al.2009). Multiple organs failure including cardiovascular system in avian influenza disease is proposed as the most likely cause of death during these outbreaks.

References:-

1- International des Epizooties. Avian influenza. World Organization form Animal Health.2.03.04AI.pdf.
2- Lamb, R.A. and P.W. Choppin, The gene structure and replication of influenza virus. Annu Rev Biochem, 1983. 52: p. 467-506.
3- Tong, S., et al., New world bats harbor diverse influenza A viruses. PLoSPathog, 2013. 9(10): p. e1003657
4- Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, Wang D, Nelson EA, Li F. (2014) Characterization of a novel influenza virus strain in cattle and swine: proposal for a new genus in the Ortho¬myxoviridae family. mBio 5: e00031–14.
5- Senne, D.; Panigrahy, B.; Kawaoka, Y.; Pearson, J.; Süss, J.; Lipkind, M.; Kida, H.;Webster, R. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: Amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis. 1996, 40, 425–437.
6- deGraaf, M.; Fouchier, R.A. Role of receptor binding specificity in influenza A virus transmission and pathogenesis. EMBO J. 2014, 33, 823–841.
7- Pantin-Jackwood, M.J.; Costa-Hurtado, M.; Shepherd, E.; DeJesus, E.; Smith, D.; Spackman, E.; Kapczynski, D.R.; Suarez, D.L.; Stallknecht, D.E.; Swayne, D.E. Pathogenicity and Transmission of H5 and H7 Highly Pathogenic Avian Influenza Viruses in Mallards. J. Virol. 2016, 90, 9967–9982.
8- Bertram, S.; Glowacka, I.; Steffen, I.; Kühl, A.; Pöhlmann, S. Novel insights into proteolytic cleavage of influenza virus hemagglutinin. Rev. Med. Virol. 2010, 20,298–310.
9- Wu H, Peng X, Xu L, Jin C, Cheng L, Lu X, et al. Novel reassortant influenza A(H5N8) viruses in domestic ducks, eastern China. Emerg Infect Dis. 2014;20:1315. http://dx.doi.org/10.3201/ eid2008.140339.
10- Lee DH, Sharshov K, Swayne DE, Kurskaya O, Sobolev I, Kabilov M, et al. Novel reassortant clade 2.3.4.4 avian influenza A(H5N8) virus in wild aquatic birds, Russia, 2016. Emerg Infect Dis. 2017;23:359–60. http://dx.doi.org/10.3201/ eid2302.161252.
11- Lee DH, Torchetti MK, Winker K, Ip HS, Song CS, Swayne DE. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. J Virol. 2015;89:6521–4.
12- Luna,G.L.(1968). Manual for histologic staining methods of the armed forces institute of pathology. McGrow-HillBookCompany ,New york, USA 3rd ed.
13- Kapczynski DR, Pantin M, Guzman SG, Ricardez Y, Spackman E, Bertran K. Characterization of the 2012 highly pathogenic avian influenza H7N3 virus isolated from poultry in an outbreak in Mexico: pathobiology and vaccine protection. J Virol. 2013;87(16):9086-96. doi: 10.1128/JVI.00666-13.

14- WHO. Recommended laboratory tests to identify avian influenza A virus in specimens from humans. Geneva .June. 2005; ests2.6. Chen W.

15- Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P. Predicting the global spread of H5N1 avian influenza. ProcNatlAcad Sci.2006; 103(51):19368–19373.

16- Kim HR, Kwon YK, Jang I, Lee YJ, Kang HM, Lee EK. Pathologic changes in wild birds infected with highly pathogenic avian influenza A (H5N8) Viruses, South Korea, 2014. Emerg Infect Dis. 2015;21(5):775-80. doi: 10.3201/eid2105.141967.

17- Swayne DE. Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. Avi Dis. 2007;51(1Suppl):242-9. doi: 10.1637/7763-110706-REGR.1.

18- Puren, Adrian; Gerlach, Jay L.; Weigl, Bernhard H.; Kelso, David M.; Domingo, Gonzalo J. (2010). "Laboratory Operations, Specimen Processing, and Handling for Viral Load Testing and Surveillance". The Journal of Infectious Diseases. 201: S27–36. doi:10.1086/650390. PMID 20225943.