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MOLECULAR, SEROLOGICAL AND PATHOLOGICAL STUDY ON BROILER BREEDERS AFFECTED WITH INFECTIOUS BRONCHITIS VIRUS

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Abstract

The study was conducted to detect the IBv. specific antibodies in serum and IBv. RNA in cecal tonsil and oviduct tissue of broiler breeder and backyard Iraqi local chickens in AL-Dewaniya province, the technique have been used throughout the study was antigen rapid test , Enzyme Linked Immunosorbent Assay (ELISA), While real time reverse Transcription polymerase chain Reaction (rRT-PCR) was used to detect IBv. genome ,in addition study the histopathological changes of grossly affected oviduct, This is the first study of its kind in this province.

Totally 90 serum samples were collected of 60 clinically ill commercial broiler breeder and 30 from backyard Iraqi local chickens, antigen rapid test showed that 14.44% were positive the rest of samples 85.56% were negative for broiler breeder, while, all local chickens were negative . ELISA results showed 45 (75%) serum samples were positive to specific IBv. antibodies ,while15(25%) were negative,but local chickens reveald 7(23.33%) was positive while, 23(76.66) samples were negative

The total RNA were extracted from tissue prepared for rRT-PCR, detection was performed using primers IBV'5GU391 , IBV5'GL533 and IBV5' G Probe , the results were performed on total 52 samples collected from clinically infected broiler chickens(45 Elisa positive) and from backyard Iraqi local breed(7 Elisa positive) , 41(78.84%) samples were positive ,35 from broiler breeders and 6 from local chick The histopathological study showed infiltration of lymphocyte and congestion of blood vessels and oviduct wall thickness.

Key words: broiler breeder, rRT-PCR, molecular detection of IBv., Infectious bronchitis virus, histopathology of IBv. Infection

Introduction

There is a wide world concern about the spreading and frequent incidence of Avian Infectious Bronchitis (IBv.) in the poultry sectors ,now days Infectious Bronchitis Virus is a predominating respiratory virus of chickens in almost all countries that raise different breed race or strains of commercial chicken flocks **(1),** the disease manifested in hens with no specific signs , respiratory signs, unsatisfactory weight gain , affect egg production and distortion of egg shell quality (ugly eggs) **(2).** The virus multiplication occurs in the oviduct and testes of infected birds of both sexes subsequently reduced egg production and fertility(**3).**In layers the disease has been associated with morbidity of 2% and weekly mortality 12% **(4).**The most commonly used vaccine strains are of the Massachusetts and Connecticut antigenic groups, which is somewhat satisfactory in controlling IB disease and losses in production with different levels of protection (**1**). Although the presence of IBv. in vaccinated chickens has been reported **(5)**.However, as a consequence of recombination and mutation of the genome of the virus (**6**) the continuously emanation or emergence of of new IBv. variants as more than 50 serotypes of the virus have been reported in the world wide **(1).** especially the IBv. variants which had become prevalent in these days in many countries of the world such as middle-east like Iraq **(7),** Egypt **(8)** and Jordan **(9)**.There for it remains a problem for the poultry industry and vaccine producers **(6)**.

The objective of the present study was to determine the presence of field isolate of IBv. directly by molecular technique and indirectly by serological method in vaccinated breeder layers and non vaccinated backyard chickens , in addition to recording the clinical signs, post mortem lesions and histopathological changes.

Materials and Methods

Samples

90 serum samples were collected from 60 clinically infected vaccinated breeders and 30 non-vaccinated local backyard chickens (AL-Dewaniya city) during winter months (January , February and March) , this birds were suffering from respiratory distress and having a mortality rate above the normal range accompanied by decrease in egg production (no peak in egg production in broiler breeders) , the commercial breeds are vaccinated with IBv. killed vaccine and boosted with repeated live vaccine, while no such vaccination in backyard bird had been done, the age range of examined birds between 18 to 25 weeks. Blood samples were collected (3ml) obtained from wing by sterile syringes and poured in to a clean plane tube

without anticoagulant and centrifuged at 2000 rpm for 5-10minute,the serum volume was ranging from0.5 to 0.75 ml . The serum was separated and stored in multiple marked sterile epindroff tubes at (4˚c) for ELISA test, while, cecal tonsil and oviduct tissues were collected, 45 samples from broiler breeder and 7 from local chickens for RNA extraction and histopathology, stored in deep freeze and formalin 10% respectively .

Anigen Rapid IBv. Ag Test Kit (BioNote, Inc.Korea).

is a chromatographic immunoassay for the qualitative detection of Infectious Bronchitis Virus antigen in avian trachea, kidney or feces sample.

anigen test kit has a letter of (C) as control line and (T) as test line on the surface of the device.

Materials provided (10 Tests/Kit)

1) Anigen Rapid IBv. antigen test devices x 10

2) Sample collection tubes containing assay diluent x 10

3) Sample collection swabs x 10

4) Disposable droppers x 10

5) Instruction for use x 1

The procedure of the test is according to the manufacturers.

ELISA technique

IBv. ELISA Kit(Synbiotics-USA) was used for the detection of specific antibody to IBv. in the serum samples according to manufacturer's instructions. Briefly, 100 µl of diluted samples were added to the pre-coated plate and incubated at room temperature for 30 min. Appropriate positive and negative control was also included. After aspirating the liquid content of all wells, the wells were washed with distilled water. 100 µl of Anti-Chicken IgG Peroxides conjugate was added into each well and the plates were incubated at room temperature for 30 min. After washing procedure, 100 µl the substrate reagent was added into each wells and incubated at room temperature for 15 min. To stop the reaction, 100 µl of Stop solution was added into each well. The relative level of antibody in the sample was determined by calculating the Sample to Positive (S/P) ratio. The endpoint titers were calculated using the equation described by the manufacturer. Serum samples with S/P ratio of lower than or equal to 0.2 were represent a negative result and those samples with S/P ratio greater than 0.20 (titer >396) were considered positive.

RNA Extraction

The RNAs were extracted from cecal tonsil and oviduct tissues using Accuzol reagent (Bioneer- South Korea) ,Briefly, at first , Homogenize tissue sample in 10-20 volumes Accuzole , Then add 200 µl of chloroform per 1ml Accuzole and shake vigorously for 15 seconds, then incubate the mixture on ice for 5 min. After that centrifuge at 12000 rpm for 15 min. at 4˚C,Transfer the aqueous phase to a new 1.5ml tube and add equal volume of isopropyl alcohol ,Mix by inverting the tube 4-5 times and incubate at deep freeze for 10 min, Then centrifuge at 12000 rpm for10 min. at 4˚C then remove the supernatant and add 1ml of ethanol and mix well by vortexing then centrifuge at 12000 rpm for 5 min. then remove the supernatant , Dry the pellet at last dissolve RNA in RNAase –free water and incubating for 10 min. at 55 to 60˚C.

rRT-PCR

The IBv. detection primers and prope used in this study have been evaluated previously by **(10)** . The primers and prope sequence are as follows: IBV'5GU391 (GCT TTT GAG CCT AGC GTT), IBV5'GL533 (GCC ATG TTG TCA CTG TCT ATTG), and (CAC CAC CAG AAC CTG TCA CCTC) . The RT-PCR produces a 143 bp fragment common to all IBV in positive samples of the 5'UTR gene. One step RT-PCR was performed by using amplification of target RNA. According to the manufacturer's instructions. Briefly, 50µl reaction volume per sample was prepared by adding 10 µl of viral RNA template , 3µl (25 pmole /µl) of each downstream and upstream primers, 3 µl (25 pmole) of probe and 31µl of DEPC water Prepare after prepare the RT-PCR plate which contain (96) well and distribute these reagents (Master- Mix) in volume (50µl) in each well, seal the tubes or plate using optical adhesive film for RT-PCR then completely mix by vortexing and centrifuge at 3,000 rpm, for 2 min then load the plate onto RT-PCR instrument and inside this apparatus take place three step for each step specially temperature and time (table 1) ,then give florescent light this light will be explained by the apparatus and send this information to the computer

Table(1): Protocol steps for primer in RT- PCR assay.

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Step	Condition	Cycle
Reverse transcription	50-70˚C min	**1**
Pre-Denaturation	95˚C,3-5 min	**1**
Denaturation	95˚C, 5-80 sec	**40-45**
Annealing/Extension/Detection	55-60˚C,30-35 sec	

Histopathological Examination:

A thin slide of 5um thickness were prepared from oviduct of birds with pathological lesions. Preparation of slides was performed according to **(11)**. After 72 hrs of the fixation, the specimens were washed with tap water and then processing was routinely done with a set of upgrading alcoholic concentration from 70% to absolute 100% for 2 hrs in each concentration to remove water from the tissues, then clearance was done by xylol, then the specimens were infiltrated with semi-liquid paraffin wax at 58 °C on two stages, then blocks of specimens were made with paraffin wax and sectioned by rotary microtome at 5µm . All tissues were stained with Hematoxylin and Eosin (H & E) stain and the histopathological changes were observed under light microscope.

Statistical Analysis

The results were analysed by SPSS program, version 17 software (2010). Analytical test including the Chi-square test and T-test were used and values less than 0.05 were considered as significant.

Results

Rapid test

The results in (Table. 2a) showed that there is 14.44% of samples was positive, (The presence of two color bands T and C , indicates a positive result.) ,while 85.56% was negative (The presence of one band C indicates a negative result.), although (Table. 2b) reveals the local chickens were totally negative .

ELISA assay

60 serum sample collected from vaccinated symptomatic broiler breeder and 30 from non vaccinated backyard local chickens, 45(75%) serum samples were positive and remaining serum samples 15(25%) were negative (Table2a),while the positive local chickens were 7(23.33%) and 22(73.33%%) were negative (Table2b), with ELISA assay, there were not any significant difference ($P<0.05$) of positive samples between the male and female and the antibody titer, but clearly there is a significant differences with the titer of positive 8030 and negative 308 samples, same results recorded with respect to local chickens.

Real Time Reverse transcriptase Polymerase Chain Reaction (rRT-PCR)

the results were performed on 52 tissue samples, 41 samples 78.84%, were positive (45 samples from broiler breeder and 7 from local chickens) the amplification plot of one step Reverse Transcription Real-Time PCR (RT-qPCR) that explains the positive results are showed in (Fig.1.).

Histopathology

Magnum, there is with high infiltration of inflammatory cells particularly lymphocytes in the interstitial tissue and abnormal columnar epithelium of follicles loss of cilia(Fig.2), while, isthmus showed desquamation of epithelium in follicles with congestion of inflammatory cells and high infiltration of inflammatory cells(Fig.3), in the epithelium of uterus there is sever degeneration and infiltration of inflammatory cells, with congestion and hemorrhage , (Fig.4)

Discussion

Rapid test

With respect to the results of rapid detection of IBv. antigen ,the high percentage of negative results make us ask questions ? why the negative results is higher than positive comparing to ELISA assay below , is there limitations of the test , according to the manufacturer the test is very accurate in detecting IBv., although occurrence of low false results have been recorded, this may be refer there is no enough IBv. particles in the taken samples (low virus load) or disease did not happen recently , another reason such as condition of transportation or storage of the kit could be considered.

ELISA assay

although ELISA, it tends to lack specificity, sensitivity and may be unable to detect all strain or types of IBv. **(12, 13, 14).** still ELISA technique is a appropriate, dependable and somewhat easy, fast and simple method used for virus diagnosis or for detect specific antibodies for viral infection such as IBv. **(15)** or viral vaccine. The technique or results, based on high antibody titers in the serum by using plate coated with inactivated virions as antigen. The antibodies can be detected after (7-10) days from challenge or infection or vaccination and this humoral immune response continue arise until four weeks after infection at which the level of antibody will stabilize**,** the serological methods which used for detection of antibody titers like ELISA is depend on some important factors such as virulence of IBv. strains, nutrition, chickens health, and concurrent diseases **(16)**, the high titer of Abs of positive samples may be due to repeated vaccination, or birds exposed for subclinical infection during the rearing or breeding period.

It's important to say that many researchers have shown that the presence or absence or the titer level of serum antibody against IBv. does not correlate with protection, and as previously mentioned that vaccinated chickens against IBv. may partly protects chickens but it could not be completely protect vital organs infection (**17**).

Table(2a): Mean Ab titers of serum samples tested with ELISA of broiler breeder.

samples	Total no. of Serum sample	No. of positive sample	Abs titer of positive sample	CV%	No. of negative sample	Abs titer of negative sample	CV%	Rapid test
Total	60	45^{A} 75 %	8030^{C}	77.55	15^{B} 25 %	308^{D}	108	pos+14.44% neg-85.56%
Male	15	10^{a} 66.67%	7989^{a}	65.7	5^{a} 33.33%	316^{a}	87	-
Female	45	35^{a} 77.78%	8072^{a}	89.4	10^{b} 22.22%	300^{a}	129	-

Horizontal and vertical rows and columns with different subscripts were significantly differed at ($P<0.05$).

Table(2b): Mean Ab titers of serum samples tested with ELISA of local chickens .

samples	Total no. of Serum sample	No. of positive sample	Abs titer and CV%	No. of negative sample of Local breed	Abs titer and CV%	Rapid test
Total	30	$7/30^{A}$ 23.33%	1039^{C} (CV%111)	$23/30^{B}$ 76.66%	285^{D} CV%96.5	-
Male	5	0	-	$5/5^{a}$	272^{a} (CV%103)	-
Female	25	7 23.33%	1039 (CV%111)	$18/25^{b}$	298^{a} (CV%90)	-

Horizontal and vertical rows and columns with different subscripts were significantly differed at ($P<0.05$).

The result of the present study was in agreement with **(18),** he concluded that broiler breeder flocks in Jordan that had respiratory disease, 70% of flocks were positive to IBv. of which it's relatively similar with the result obtained by our study which is (75%), in another study **(19)** who used HI and ELISA to determine the rate of IBv. antibodies titer in broiler breeder with respiratory symptoms was (82.43%,). **(20)** studied the sero-prevalence of IBv. in commercial poultry in Pakistan showed that 88% of the flocks were sero-positive for M-41 antibodies and the results of these 2 studies it's higher than our study.

The different antibody titers of IBv. exhibited by different studies indicated different sero-prevalence and variable epidemiological features among examined chickens in different countries, which revealed different exposure rates of chickens to virus or different pressure load of virus in the confined poultry environment also frequent raising chickens throughout the year with high density maybe coincides with the increase in the incidence and spread of the disease among poultry houses and farms ,What should be critically considered is presence of different strains of the virus , and we should remember that available vaccination is Maas. Type as a major vaccines used in protecting poultry **(21).** Also this variable results could be referred to or attributed to different climatic conditions , a dusty and dry climate that aid the transmission of IB virus **(19)** such climatic conditions do exist in this province. According to the (Table 2a,b) the uniformity of the ELISA titers is a good calibration of determining the

suspected infected flocks. the CV% of high antibody titer was 77.5, 108, and low antibody titer was111, 96.5 of broiler breeds and local chickens , respectively. It means that the uniformity of these birds was not satisfactory because the optimum CV% is below 50 , this results may refers to that, the birds either have incomplete or non-uniformity of vaccine administration, or have no vaccination or poor vaccination response or have early exposure to IB virus.

Real Time Reverse transcriptase Polymerase Chain Reaction (rRT-PCR)

The rRT-PCR is a powerful technique in detecting several RNA viruses **(22, 23, 24).** , this technique performed on IBv. extracted from cecal tonsils and oviduct tissues (for our knowledge little researches has been done regarding the IBv. with the oviduct of the laying hen) in our study was promising as a diagnostic tool as showed in Fig.1.

rRT-PCR is commonly used for the detection of IBv. because it considered highly specific and sensitive technique to detect viral RNA directly from a clinical sample. When RT-PCR is used to amplify the glycoprotein of IBv., it can coupled with restriction fragment length polymorphism or nucleic acid sequencing to identify the type of the virus **(25,26,27,28,29),** In this study the one-step real time RT-PCR technique was used for the detection N protein gene of IBv. in the tissue samples (cecal tonsils and oviduct), The results of RT-PCR indicated the viral RNA load is recordable or satisfactory to read because most of these results located between C.T (20.31) to C.T(42.99) whereas the control sample located in C.T (35) and any sample results below threshold line considered negative result, these results may indicate infection of these birds with IBv., in this study detection IBv.RNA in the tissue of vaccinated chickens, claiming that IBv. vaccine did not fully protect against infection that the antibody titer of vaccinated chickens may not have been enough to protect them from infection, virus could be mutant of vaccine strain, or chickens may be infected simultaneously with several type of IBv. **(30).**

The results of this study were corresponding with **(31)** who had examined broilers breeder flocks suffering from respiratory signs ,(75%) of examined samples for the presence of IBv. by RT-PCR were positive.

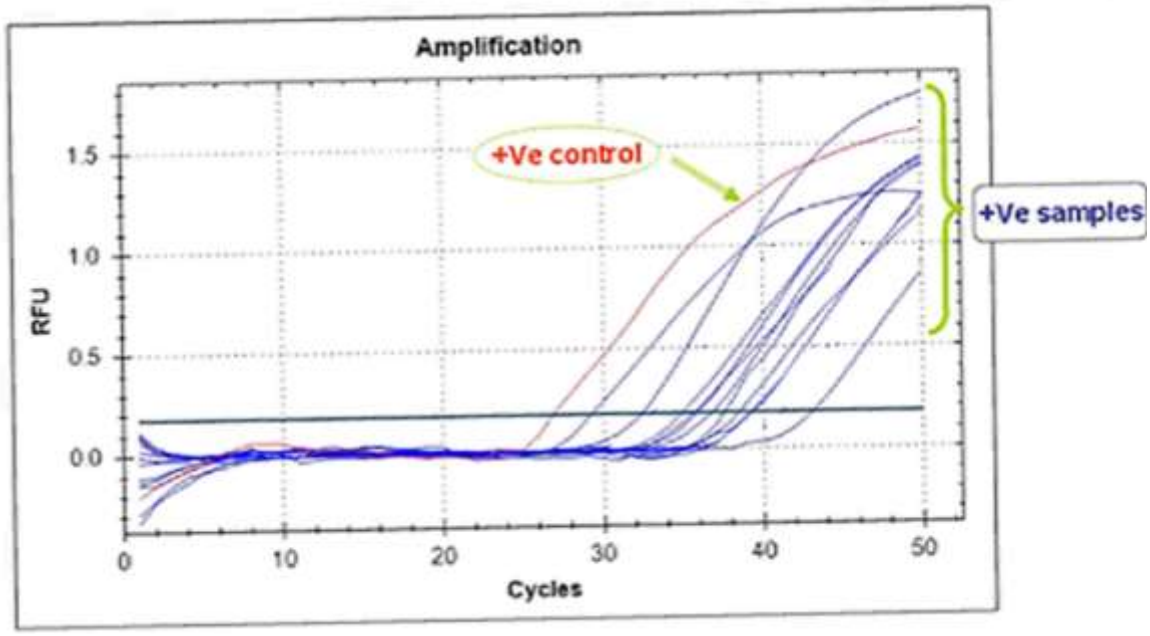

Figure (1): Amplification plot of one step Reverse Transcription Real-Time PCR (RT-qPCR) that explains the positive results

Histopathology lesions

Our broiler breeders under histopathological examination showed mild respiratory signs , slight decreased of feed consumption and egg production Misshaped shell, with poor internal quality , while slight or no signs were noticed or observed in local chickens except low egg production and some labeled hens were with no production or false layers .

The impacts of IBv. on production of eggs has been reported by several scientific workers**(32)**,although associated changes(grossly or histopathology) in the different parts of the oviduct of adult layer birds had been elucidated differently , in our investigation the post-mortem examination revealed cystic ovary of some birds (Fig.5) and congestion of the whole oviduct (Fig.6) and occasionally others showed waved shortened oviduct.

These histopathological lesions in this study mentioned previously in same line with **(33)**, who showed infiltration of lymphocytes other mononuclear cells, plasma cells and heterophilis and congestion of vessels in the oviduct. While, experimental IB infection of the oviduct of mature hens resulted in decreased height and loss of cilia from epithelial cells; dilation of the tubular glands; infiltration by lymphocytes, other mononuclear cells, plasma cells, and heterophils; and edema and fibroplasia of the mucosa of all regions of the oviduct **(34)**.

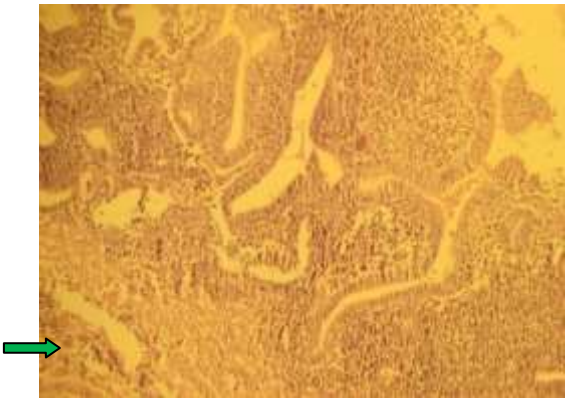

Fig. (2): magnum. High infiltration of inflammatory cells particularly lymphocytes and macrophages in the interstitial tissue (green arrow). 10X H&E.

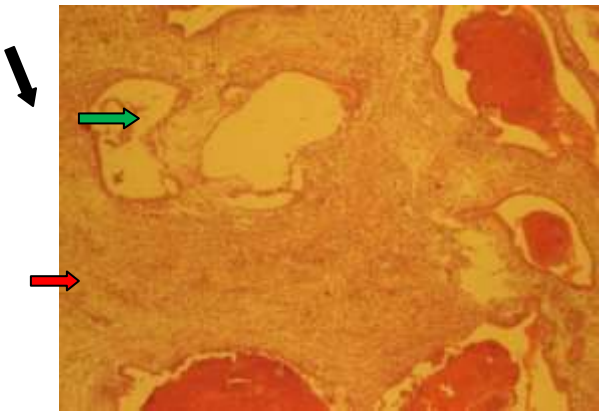

Fig. (3): isthmus. There is desquamation of epithelium of follicles (blak arrow) and congestion(green arrow) and sever infiltration of inflammatory cells particularly lymphocytes in the interstitial tissue(red arrow) .10X H&E.

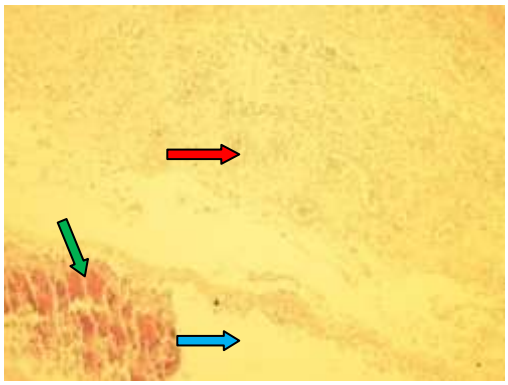

Fig. (4): uterus. degeneration (blue arrow) with infiltration of inflammatory cells in the epithelium of uterus(red arrow) and heamorrhage (green arrow).10X H&E.

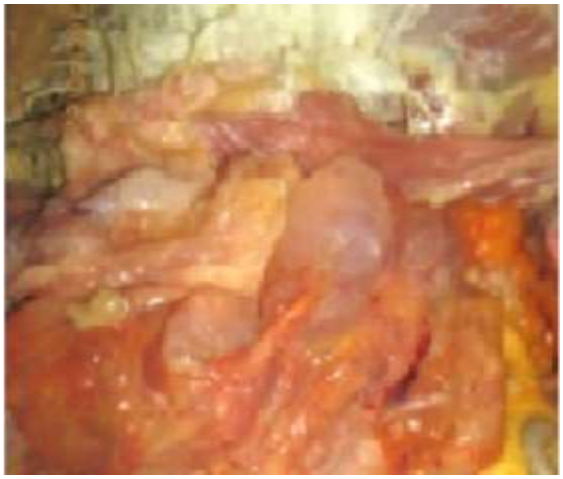

Fig.(5):Cystic ovary

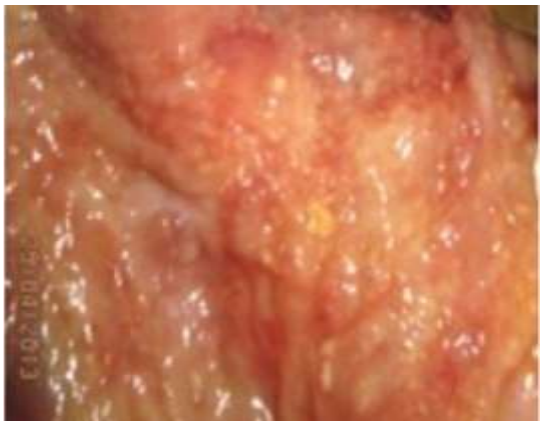

Fig.(6): congested oviduct

(uterus area)

References

1-Cavanagh., D. (2003). Sever acute respiratory syndrome vaccine development: experiences

of vaccination against avian infectious bronchitis corona virus. Avian Pathol.,32:567-

582.

2- Cavanagh, D. and Naqi, S. (2003). Infectious bronchitis, In diseases of poultry, Saif, Y.

M., Barnes, H. J., Glisson, J. R., Fadly, A.M.,McDougal, L.R. and D.E.Swayne, eds.

(Ames, Iowa: Iowa state university Press), pp 101-119.(Cavanagh D, Naqi SA. Infectious bronchitis. In: Saif YM, ed. Disease of Poultry. Ames, Iowa: Iowa State Univ Pr, 2003: 101–119.).

3- Boltz DA, **Nakai M, Bahra JM**. **(2004)**. Avian infectious bronchitis virus:A possible cause

of reduced fertility in the rooster. Avian Dis.,48:909–915.

4- Zanella, A.; Lavazza, A; Marchi, R.; Moreno Martin, A.; Paganelli F.(2003).Avian Infectious Bronchitis: Characterization of New Isolates from Italy. Avian Dis.,47: (1)180-185.

5- Alvarado, I.R.; Villegas, P.; El- Attrache, J. and Brown, T.P.(2003). Evolution of the protection conferred by commercial vaccines against the California 99 isolate of infection bronchitis virus. Avian Dis.,47: 1298-1304.

6- Nix, WA, Troeber DS, Kingham BF, Keeler CL Jr, Gelb J Jr. (2000). Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Dis.,44:568–581.

7- Al-Husaairaji, F.F. (2005). Isolation, identification and pathogenesis of infectious bronchitis virus in broilers in Northern area of Iraq. M. Sc.Thesis College of Veterinary Medicine –Mosul University – Iraq.

8- Mahgoub, K.M.; Khaphagy, A.; Bassiouni, A. and Negwa, R.S.(2010). The prevalence of infection bronchitis (IB) outbreaks in some chicken farms. II.Molecular characterization of field isolate IB virus. J. Am. Sci.,6:71-93.

9- Roussan.D.A., Totanji, w.s. and Khawaldch, G.Y. (2007). Molecular subtype of infectious bronchitis virus in broiler flocks in Jordan. Poult. Sci. Asso.,87:661-664.

10- Callison,S.A.; Hilt,D.A.; Boynton, T.O; Sample, B.F; Robison, R.; Sawayne, D. E. and Jachwood, M.W.(2006). Development and evolution of real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J. virol. Meth.,138:60-65.

11- Luna, L.G.(1986). "Manual of Histological Staining Method of the Armed Force Institute of Pathology ". 3 Ed. McGraw-Hill, New York.

12- Karaca, K.; Naqi, S. and Gelb Jr, J. (1992). Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis., 36(4), 903-915.

13- Karaca, K. and Naqi, S., (1993). A monoclonal antibody blocking ELISA to detect serotype- specific infection bronchitis virus antibodies. Vet.Microbiol.,34:249-257.

14- Naqi, S.A.; Karaca, K. and Bauman, B. (1993). A monoclonal antibody- based antigen capture enzyme –linked immunosorbent assay for identification of infectious bronchitis virus serotype. Avian Pathol.,22:555-564.

15- Wing, C.H., Hong, C.C. and Seak, J.C.H. (2002). An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. Vet. Microbiol.,85:333-342.

16- Chen, H.; Coote, S. and Hiscox, J.A. (2003). Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. Avian Pathol., 32:519-526.

17- Faramarzi Saman, Peyman Bijanzad, Vahid Javaherzadeh, Hamed Moomivand, Ehsan Stabraghi, Elias Nouri Dehnavi, Armin Ghaedi, Ali Zarghami (2014) Evaluation of two different infectious bronchitis vaccination programs in broiler breeder chickens Int. J. Bio.sci., 5: (4) 210-216.

18- Gharaibeh, S.M.,(2007). Infectious bronchitis virus serotype in poultry flocks in Jordan. Prev. Vet. Med., 78:317-324.

19- Hadipour, M.M.; Habibi, G.H.; Golehin, P.; Hadipourfard, M.R. and Shayanpour, N.(2011). The role of avian infectious influenza, Newcastle diseases and infectious bronchitis viruses during the respiratory disease outbreak in commercial broiler farms of Iran. Int. J. Anim. Vet. Adv., 3:69-72.

20-Ahmed Z,; Naeem K. and Hameed A.(2007). Detection and seroprevalence of infectious bronchitis virus strain in commercial poultry in Pakistan. Poul. Sci., 86:1329-1335.

21- Seyif Abed Shapouri, M.R.M.; Mayahi, S.; Charkaher and Asasi, K. (2002). Serotype identification of recent Iranian isolate of infectious bronchitis virus by type-specific multiplex RT-PCR. Arch. Razi Ins., 53:79-84.

22- Jackwood, M.W.; Kwon, H.M. and Hilt, D.A.(1992).Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Dis.,36:403-409.

23- Adzhar, A., Shaw, P., Briton, P. and Cavanagh, D. (1996). Universal oligonucleotides for detection of infectious bronchitis virus by the polymerase chain reaction. Avian pathol. 25:817-836.

24- Falcon, E.; Dqamore, E.; Di Trani, L.; Sili A. and Tollis M. (1997). Rabid diagnosis of avian infectious bronchitis virus by polymerase chain reaction. J. virol. methods., 64:125-130.

25-Cavanagh, D.; Davis, P.; Cook, J. and Li, D.(1990). Molecular basis of the variation exhibited by avian infectious bronchitis corona virus (IBV). Adv. Exp. Med. Biol.,276: 396-372.

26- Kwon, H.M.; Jakwood, M.W.; Brow, T.P., and Hilt, D.A.(1993). PCR a biotin-labeled DNA probe for detection of IBV in chickens, Avian Dis., 37:149-156.

27- Jackwood, M.W.; Yousef, N.M. and Hilt, D.A.(1997). Further development and use of molecular serotype identification test for infectious bronchitis virus. Avian Dis.,41(1).105-110.

28- Keeler, C.; Reed, K.L.; Nix, W.A. and Gelb, J.(1998). Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis.,42(2), 275-284.

29- Kingham, B.F.; Keeler, C.L.; Nix, W.A.; Ladman, B.S. and Gelb, J.(2000). Identification of avian infectious bronchitis virus by direct automatic cycle sequencing of the S-1gene. Avian Dis.,44(2), 325-335.

30- Stachowiak, B., D. W. Key, P. Hunton, S. Gillingham, and E´ . Nagy, (2005). Infectious Bronchitis Virus Surveillance in Ontario Commercial Layer Flocks2005 J. Appl. Poult. Res.,14:141–146.

31- Roussan, D.A.; Khawaldeh, G.Y. and Sahahen, I.A.(2009). Infectious bronchitis virus in Jordanian chickens: Seroprevalence and detection. Can.Vet.J., 50:77-80.

32- Mahgoub, KM., Bassiouni AA., Manal A, Afify., and Nagwa, RS. (2010). The prevalence of infectious bronchitis (IB) outbreaks in some chicken farms. I. Spotlight on the status of IB outbreaks in some chicken flocks. J. Am. Sci.,6(9):57-70.

33- Purcell, D.A.; Than, V.L. and Surman, P.G. (1996). The histopathology of infectious bronchitis in fowls infected with a nephrotropic 'T' strain of virus. Aust. Vet. J.,52:85-91.

34- Riddell, C. (1987). Avian Histopathology. American Association of Avian Pathology: Kennett Square, PA.