ISOLATION AND IDENTIFICATION OF INFECTIOUS BRONCHITIS VIRUS AND EXPERIMENTAL INFECTION IN BROILERS

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ABSTRACT

Poultry in iraq especially broilers showed high percentage of mortality, some of them reached (70%), with severe respiratory signs, depreassion and loss of body weight. Also nephritis with urolethiasis. Infectious bronchitis virus (IBV) was the suspected infections agent. So that, this study was conducted to diagnose IBV in broilers through estimation of IBV antibody by Enzyme Linked Immunosorbent Assay (ELISA) technique through collecting blood samples from infected farms and isolate the virus using one step real time RT-PCR and chicken embryo fibroblast tissue culture (CEF). The isolated IB virus was used to carry on an experimental infection in broiler chicks. Lung, trachea and kidney samples were collected for histopathological study. ELISA results showed very high titers of IBV antibodies after (14) day post clinical signs appearance. rRT-PCR results were positive for the samples (trachea, lung and kidney) collected in the beginning of the disease. Tissue culture results showed pathological tissue changes after inoculation with the isolated virus. Twenty broiler chicks were used for experimental infection on day (22). Clinical signs appeared (36 hr) post infection with (32%) mortality and histopathological changes characterized by lymphocytic infiltration and congestion in trachea, lung and kidney.

INTRODUCTION

Avian infectious bronchitis disease is an acute, highly contagious viral disease of the respiratory system in chickens and cause high mortality (80%), poor weight gain in broiler and also associated with nephritis (Dhinakar and Jones, 1997 ; Butcher *et al.,* 2002).

In chicks it is characterized by depression, gasping, coughing and nasal discharge with tracheal, lung congestion and kidney swollen (nephritis). In layer it is characterized by degeneration of the ovary and swollen oviducts, drop in eggs production and poor quality eggs (Cavanagh and Naqi, 2003).

It is RNA virus, single stranded, positive sense, encoding several proteins which are associated with RNA replication and transcription, has four proteins (S protein, E protein, M protein and N protein) (Boursnell *et al.,* 1984). In the same time there are more than (30) known serotypes within IBV recognized

worldwide, some of these strains consider variant strains (Casais *et al.,* 2003). IBV infections can be diagnosed by detecting the appearance or rise in titer of IBV specific antibodies which appeared (10-14) days after infection (Pei and Collisson, 2005). The major uses of RT-PCR tests are IB virus identification and typing (sequencing) its application in the understanding of epidemiological investigations during IBV outbreaks, which make the diagnostic fast and also reliable (Capua *et al.,* 1999 ; Kingham *et al.,* 2000).

The aims of this study were to:

1- Diagnose IBV in broilers through estimate of IBV antibody titer in suspected infected broiler farms by use the ELISA test.
2- Try to isolate the causative agent IBV on tissue culture (CEF) from trachea, lung and kidney samples.
3- Confirm the diagnosis of isolated IBV by rRt- PCR technique from trachea, lung and kidney samples.
4- Confirm the suspected IB infection by experimental infection and histopathological study.

MATERIALS AND METHODS

ProFLOK® IBV ELISA Kit (Synbiotics–USA) was used in this study. Tissue samples (trachea, lung and kidney) were collected from ten broiler farms suffered from respiratory signs with kidney lesion and high mortality. Samples placed in containers with ice and stored in (-4 °C), all these farms did not vaccinated with any IBV vaccine.

Blood samples were collected from 9 birds of each of the ten farms after 14 days of infection to be used in ELISA test for detection of IBV antibodies by using IBV antibody test kit (Synbiotics-USA).

Chicken embryo fibroblast cell (CEFC) prepared according to Paul (1970). Primary cultures from CEF were prepared according to Karel and Purchase (1989). Ten falcons were prepared containing (CEFCc) each sample for specific falcon and when cells became monolayer cells, the growth medium was infused and inoculated (CEFCc) with IBV (taken from sample) and replaced the growth medium with maintenance medium and incubated at (37 °C) with testing all falcons daily under the Inverted microscope for (7 days). In the first blind passage of virus after seven days all samples did not produce any cytopathic effect (CPE) in these ten culture falcons after that all these falcons with cell cultures were frozen in (-80° C) and the second blind passage started by preparation another chicken embryo fibroblast culture cell in ten falcons and the virus was taken from the old culture (frozen culture) by thawing after that

propagation the virus in new culture and incubated at (37 °C) for seven days with daily examination,

Real Time RT-Polymerase Chain Reaction (PCR) technique was done according to Meir *et al.* (2010).

Experimental infection

Twenty broiler chicks (Breed: Ross, Origin: Turkey) housed under strict hygienic measures in a separate experimental room. They were provided with food and water, *ad libitum* with no vaccine. In age 20 day blood samples (2 ml) were collected from jugular vein of 9 chicks for detection of specific IBV antibodies by using ELISA test.

At 22 days of age, the chicken were inoculated intra-ocular and intra-nasal with 0.2 ml virus contain 1×10^6 ($TCID_{50}$) bird^{-1} and divided equally between the routes and then samples (trachea, lung and kidney) were collected from infected bird after 5 days post clinical signs appearance for histopathological study.

Histopathological Examination

Samples (trachea, lung and kidney) were collected from three sick birds in experimental infection after scarifying, the tissues were fixed in (10%) buffer formaldehyde solution immediately after collect. After 72 hours of the fixation, the specimens were processed according to (Luna, 1968).

RESULTS AND DISCUSSION

By experimental infection Cavanagh (2003) demonstrated the IBV initially infects the upper respiratory tract, and three days after inoculation of virus replication, the highest titers are found in the trachea, which may persist up to five days depend on the virus strain.

In this study the samples (trachea, lung and kidney) were collected in the early stage of disease as soon as the clinical signs were appeared from diseased chickens and all these farms were vaccinated with Newcastle vaccine and Gambaro vaccine and did not vaccinated with IB vaccine, these samples were stored at (-4 °C) to be used in rRT-PCR and cell culture.

The ELISA assay was a convenient method widely used to detect antibody response to IBV infection in broilers flock and based on high antibody titers in the serum which used plate coating with inactivated virions as antigen (Chen *et al.,* 2003). In this study blood samples were collected from ten farms did not vaccinate by IBV vaccine, these farms showed severe respiratory signs, depression, loss appetite and decreased weight gain with congested trachea, congested lung and swollen kidneys (nephritis) with mortality between 30% - 60%, after 10-14 days from samples collection blood (2 ml) was collected from

jugular vein, 6 - 10 blood samples were collected from each farm and put in cold container and ELISA (Synbiotic) was conducted and the result showed a high titer for IBV in all these farms and a low titer for Newcastle disease and Influenza disease. The means of these results alternated between 913-6876, they were 913, 1654, 5002, 2311, 1020, 2207, 4221, 6876, 902, 913 and these results considered diagnostic test for IBV infection.

The results of this study are in agreement with Hadipour *et al.*, (2011) who collected (200) blood samples of healthy chickens that ELISA technique showed high titer of IBV antibodies in chickens that did not vaccinate with IBV vaccine and the titer less than 396 considered negative and greater than 396 considered positive.

Also in agreement with Mahgoub *et al.,* (2010) who showed by experimental infection on SPF chicks after checking the maternal immunity in one day old and making the experimental challenge with field isolate then after 14 days from challenge the titer of IBV antibody was estimated by ELISA (Synbiotic), the mean of antibody titer in all those groups were alternated between 550- 746, and also in agreement with Momayez *et al.,* (2002) isolated IBV from broilers chickens showed respiratory signs and kidneys swollen with mortality, this infection took place at 2- 3 weeks and showed increased IBV antibody titers after 14 days of infection.

In this study the one-step real time RT-PCR technique was used for detection N protein gen of IBV in the tissue samples (trachea, lung and kidney) all samples gave positive results in ELISA test, all these samples gave positive result in rRT- PCR test and this result indicated the highest levels of viral RNA titer because most these results located between cycle (20.31) and cycle (30.34) and the control (+ ev) located in cycle (23.53) and any result excesses the cycle (40) considered (-ev) result and these highest levels assure infection of these farms with IBV, and all results resemble as in figure (1, 2, 3, 4, 5, 6 and 7) and table 1. The results of this study are in agreement with Handberg *et al.,* (1999) used 40-cycle rRT-PCR to detect the N protein gen of IBV in trachea tissue extracted after experimental infection of chickens with number of IBV types. Virus was detected in (75% -100%) of trachea collected at day three after inoculation with IBV. The number of sample in specific well with control (+ve) and (-ve) well and the cycle of complete cDNA extension in which the florescent was given and the apparatus explained this sign as positive or negative result.

ELISA test and RT- PCR technique showed positive results in addition to cytopathic effect in chicken embryo fibroblast culture cells (CEFCc).

In this passage showing the cytopathic effect (CPE) in sample (3, 6 and 9) was began after (72 hr) from inoculation and it was characterized by rounding of cells that infected with IBV and the cells appeared at different stages of IBV infection, and after (5) days from second passage the cells were scraped from tissue culture in the falcons and composed plaque due to IBV lead to cells destruction, all these results cytopathic effect (CPE) as in figure (1, 2 and 3).

The IBV able to replicate in chicken embryo fibroblast cell (CEFC) and also in mammalian Vero cell and BHK (baby hamster kidney) cells (Casais *et al.,* 2003). Beaudette and Hudson first cultivated IBV in chicks embryos inoculated by the chorionallantoic route (Fabricant, 2000). The use of mammalian cell cultures for primary isolation of IBV has been unsuccessful. Nevertheless, adaptation of IBV strains for good replication in a cell line is desirable, especially for studies involving virus replication and production of virus antigen (Otsuki *et al.,* 1979). Otsuki *et al.,* (1979) examined IBV replication in primary chick kidney (CK) and chick embryo fibroblast culture (CEFC) cells, and mammalian cell lines, clone 13 (BHK21 c-13). It is important to emphasize that only the Beaudette and Holte strain of IBV was able to replicate on BHK21 c-13.

The result in this study is similar to the result found by Nazerian and Cunningham (1968), he propagated IBV in chicken embryo fibroblast culture cell (CEFCc) and by using electron microscope he showed the cytopathic effect (CPE) at first passage and this CPE characterized by the cells at different stage of infection and multiplication of IBV virus appeared to be entirely intracytoplasmic and to occur most likely by budding into the cytoplasmic vesicles. Some of the cytoplasmic vesicles contained viral partials and after (40 hr) from extensive degeneration of the cell, the cells appear scraped from the dishes and formation of plaques. Otsuki and Tsubokura (1981) showed ten strains of IBV that were titrated as plaque forming units in primary chicken embryo fibroblast cells.

Figure 1. Normal chicken embryo fibroblast cells culture (normal cells). (X 40)

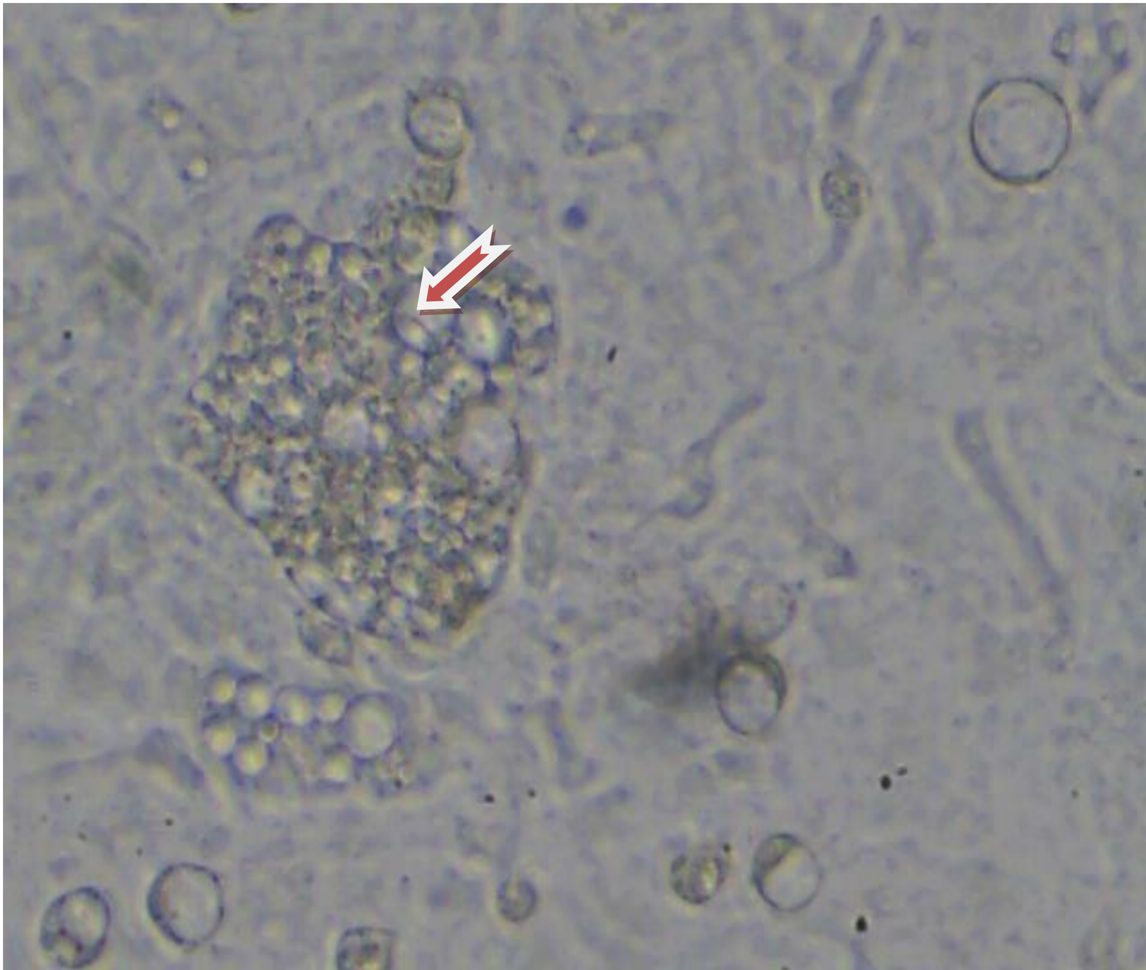

Figure 2. Cytopathic effect after (72 hr) from second passage in sample three, rounded cells that infected with IBV. (40X)

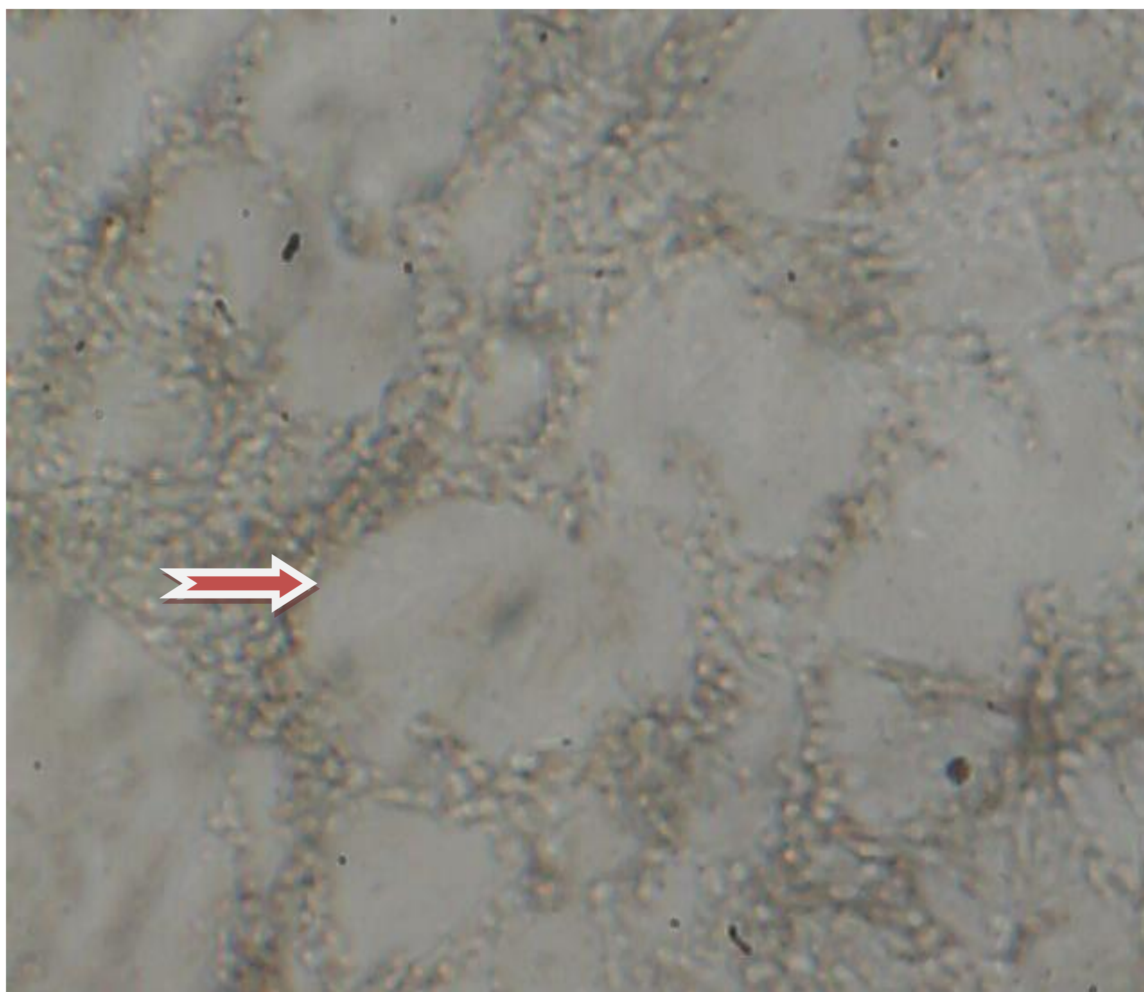

Figure 3. Shows the cytopathic effect after (5) days from second passage in sample three, characterized by plaque formation due to cell destruction by IBV. X (40)

Experimental Infection Study:

After (20) days from rearing the chicks and without giving any vaccine, blood samples (2ml) from (9) chicks randomly were collected to detect IBV antibodies (Ab) titer by ELISA and found out that mean (Ab) titer against IBV in all blood samples was zero (0), antibody titer result was similar with Mondal and Naqi (2001) who showed that maternal antibodies may control challenges for an initial period that varies from (7-14) days.

All the young chickens inoculated with a dose of (0.2 ml) of the virus isolate (1×10^6 $TCID_{50}$) exhibited respiratory signs started at (36 hours) after inoculation with virus, abstracted by ruffled feather, depression and conjunctivitis with more water intake while less feeds, watery eye, nasal discharge and gasping, and after (12) days from inoculation the mortality arrived seven from twenty chicks, in five day from experimental infection four chicks were killed to be used in histopathological examination and the gross lesion was characterized by tracheal congestion, lung congestion with pneumonia and

swollen kidney (nephritis) and the ureters were distended with urate. All these are in agreement with Zhou *et al.,* (2003) who inoculated the virus at (14) days from rearing, after two days all chicks showed respiratory signs, depression, ruffled feathers and watery dropping with increase water intake, the necropsy finding of dead birds was characterized by kidneys swollen and exhibited severe urate deposition in the end of the experiment at 14 days the mortality was 50%. Okino *et al.,* (2004) who inoculated the virus at three weeks from rearing and the respiratory signs appear after 48 hr from experimental infection with mortality.

The histopathological changes were summarized by desquamation of tracheal epithelial cells with infiltration of inflammatory cells specially lymphocyte cells, hyperplasia of epithelial cells with hemorrhage, lung showed severe infiltration of inflammatory cells mostly lymphocyte cells and severe congestion with spots of pneumonia while kidneys showed tubules degeneration, glomeriolar distention with severe hemorrhage and lymphocyte infiltration (Fig. 4).

Trachea showed deciliation, oedema, desquamation and sloughing of epithelial cells, hyperplasia and mononuclear cell infiltration of the submucosa (lymphocytes and heterophils) (Fig. 5), all these changes were observed between three and nine days after infection (Purcell and Mc Ferran, 1972). In nephropathogenic IBV strains virus replication occur firstly in the trachea and spread to the kidney and cause interstitial inflammatory response with inflammatory cell infiltration (lymphocyte and plasma cells), extensive tubular degradation and focal areas of necrosis may be seen with massive infiltration of heterophils and after (5-10) days from infection appear focal areas of uric acid precipitation in the kidney (Purcell *et al.,* 1976).

All these histopathological lesions in lung and kidney are in agreement with Zhou *et al.,* (2003) and also in agreement with Zsofia *et al.,* (2009), who demonstrated IBV (QX- like strain) concerned respiratory system and kidney.

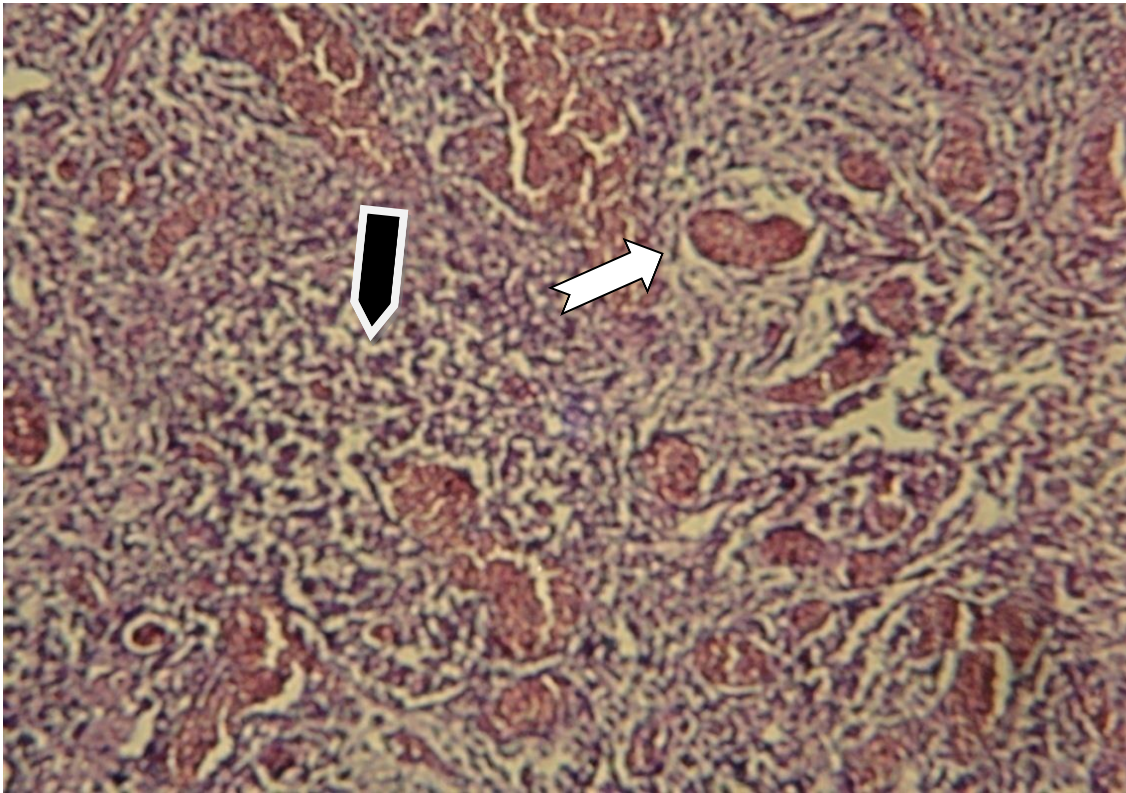

Figure 4. Lung severe congestion with hemorrhage and severe infiltration of inflammatory cells mostly lymphocyte. (Hemtoxilin and Eosin 40X)

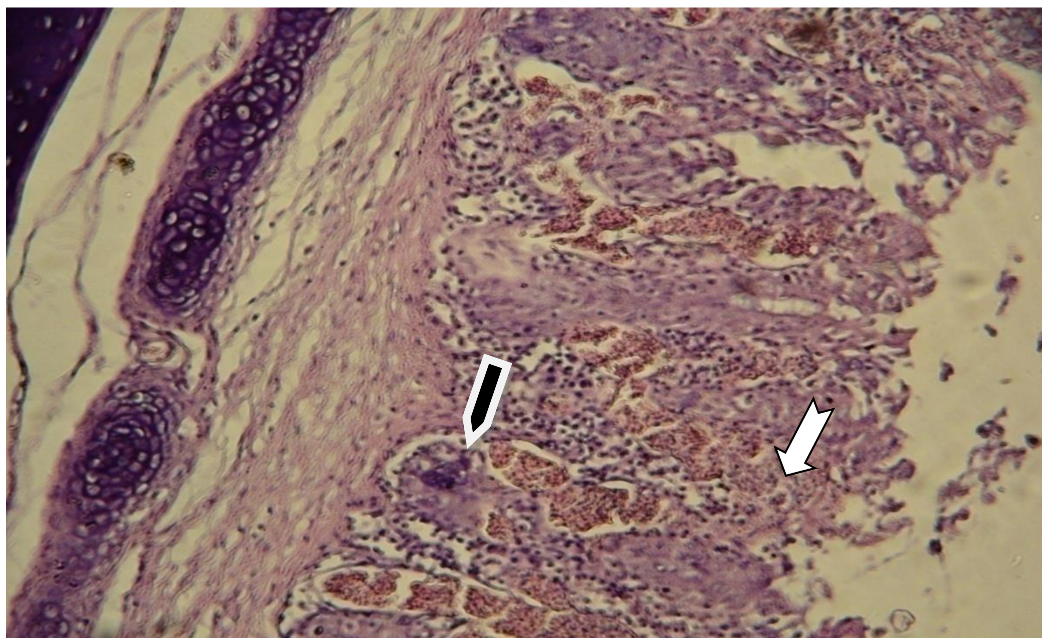

Figure 5. Trachea: severe congestion and desquamation and sloughing of tracheal epithelial cells, deciliation with inflammatory cells infiltration specially lymphocyte and hyperplasia of epithelium. (H ematoxiline and eosin 40X)

REFERENCES

Boursnell, M. E., T. D. Brown and M. M. Binns. 1984. Sequence of the membrane protein gene from avian corona virus IBV. *Virus Res*. 1(4): 303-313.

Butcher, G. D., D. P. Shapiro and R. D. Miles. 2002. Infectious Bronchitis Virus: classical and variant strains. Institute of food and agricultural science, University of Florida.

Capua, I., Z. Minta, E. Karpinska, K. Mawditt, P. Britton, D. Cavanagh and R. E. Gough. 1999. Cocirculation of four types of infectious bronchitis virus (793/B 624/I B1648 and Massachusetts), *Avian Pathol*. 28: 587-592.

Casais, R., B. Dove, D. Cavanagh and P. Britton. 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J. Virol.* 77: 9084-9089.

Cavanagh, D. 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis corona virus. *Avian Pathol*. 32: 567-582.

Cavanagh, D. and S. Naqi. 2003. Infectious Bronchitis, *In*: Saif, Y. M., H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne. (Eds.), Diseases of Poultry, Iowa, 11th edition, Ames, Iowa State University Press, 101-119.

Chen, H., B. Coote, S. Attree and J. A. Hiscox. 2003. Evaluation of nucleoprotein-based enzyme–linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. *Avian pathol*. 32: 519-522.

Dhinakar-Raj, G. and R. C. Jones. 1997. Infectious bronchitis virus: Immuno pathogenesis of infection in the chicken. *Avian Pathol*. 26: 677-706.

Fabricant, J. 2000. The early history of infectious bronchitis. *Avian Diseases*. 42: 648-650.

Hadipour, M. M., F. Azad, A. Vosoughi, M. Fakhrabadipour and A. Olyaie. 2011. Measurement of antibody to infectious bronchitis virus in indigenous chicken flocks around Maharlou Lake in Iran. *J. of animal and Vet. Advances.* 3(3): 182-185.

Handberg, K. J., O. L. Nielsen and M. W. Pedersen. 1999. Detection and strain differentiation of infectious bronchitis virus in tracheal tissues from experimentally infected chickens by reverse transcription-polymerase chain reaction. Comparison with an immunehistochemical technique. *Avian Pathology*, 28: 327- 35.

Karel, A. S. and H. G. Purchase. 1989. Cell culture methods. *In*: A laboratory manual for the isolation and identification of avian pathogens. 3rd ed. Purchase, H. G., L. H. Arp, G. H. Dormuth and J. E pearson, (eds). Kendall, Hunt, Publishing Company, Dubuque, Iowa, pp. 167-175.

Kingham, B. F., C. L. J. Keeler, W. A. Nix, B. S. Ladman and J. J. R. Gelb. 2000. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. *Avian Dis*. 44: 325-335.

Luna, L. G. 1968. "Manual of Histologic Staining Methods of the Armed Force Institute of Pathology". 3rd Ed. McGraw-Hill, New York.

Mahgoub, K. M., A. Khaphagy, A. Bassiouni, A. Manal and Nagwa, Rabie S. 2010. The prevalence of infectious bronchitis (IB) outbreaks in some chicken farms. II. Molecular characterization of field isolates IB virus. *J. American Science,* 6(9): 71-93.

Meir, R., O. Maharal, Y. Famushi and L. Simnov. 2010. Development of real-time TaqManRT-PCR ssay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. *Journal of Virological Methods,* 2(163): 190-194

Momayez, R., S. A. Pourbakhsh, M. Khodashenas and M. Banani. 2002. Isolation and identification of infectious bronchitis virus from commercial chicken. *Arch. Razi Ins*. 55: 1- 10.

Nazerian, K. and C. H. Cunningham. 1968. Morphogenesis of avian infectious bronchitis virus in chicken embryo fibroblasts, *Journal of General Virology.,* 3(3): 469-470.

Otsuki, K., K. Noro, H. Yamamoto and M. Tsubokura. 1979. Studies on avian bronchitis virus (IBV). Propagation of IBV in several cultured cells. *Archives of Virology*. 60: 115-122.

Otsuki, K. and M. Tsubokura. 1981. Plaque formation by avian infectious bronchitis virus in primary chick embryo fibroblast cell in the presence of trypsin. *Archives of virology*. 70: 315-320.

Purcell, A. A. and J. B. McFerran. 1972. The histopathology of infectious bronchitis in the domestic fowl. *Res. Vet. Sci.,* 13: 116-122.

Purcell, D. A., V. L. Tham and P. G. Surman. 1976. The histopathology of infectious bronchitis in fowls infected with a nephrotropic 'T' strain of virus. *Aust. Vet. J.,* 52: 85-91.

Zhou, J. Y., D. Y. Zhang, J. X. Ye and L. Q. Cheng. 2003. Characterization of an avian infectious bronchitis virus isolated in china from chicken with nephritis. *J. Vet. Med.,* 51: 147-152.

Zsofia, B., M. Tamas, S. Tibor, S. Eva, K. Veronika, A. Zsolt, R. Miklos and P. Vilmos. 2009. Comparison of the pathogenicity of QX- like, M41 and 793/B infectious bronchitis strains from different pathological condition. *Avian pathol*. 68: 22-29.

عزل وتشخيص فيروس التهاب القصبات الهوائية المعدي والإصابة التجريبية في فروج اللحم

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المستلخص

لوحظ في الآونة الأخيرة حدوث هلاكات بنسب عالية جدا في حقول فروج اللحم حيث وصلت في بعض الحقول إلى (70%) مصحوبة بظهور علامات تنفسية شديدة مع خمول وفقدان في الوزن والشهية كما لوحظ في بعض الأحيان التهاب الكلى وقد يكون شديد جدا مصحوبا بترسب اليوريا بشكل حصى في الكلى والحالبين.

هذه العلامات أدت إلى الأشتباه بإصابة هده القطعان بفايروس التهاب القصبات المعدي وبعد التأكد من عدم وجود مرض النيوكاسل أو انفلونزا الطيور لذلك قررت هده الدراسة لأجل تشخيص الإصابة بفايروس التهاب القصبات المعدي في دجاج اللاحم من خلال قياس معيارية الأجسام المضادة لهدا الفايروس بواسطة فحص (ELISA) ومن ثم يتم عزل وتشخيص الفايروس باستعمال تقنية الـ (RT-PCRr)، ومن ثم يتم حقن الفايروس على خلايا الزرع النسيجي لأجنة الأفراخ (CEFC). بعدها أجريت الإصابة التجريبية بفايروس التهاب القصبات المعدي المعزول وعند ظهور العلامات السريرية يتم جمع العينات (القصبة، الرئة والكلية) لأجل دراسة التغيرات النسيجية التي سببها فايروس التهاب القصبات المعدي في تلك الأعضاء.

أظهرت نتائج (ELISA) معايير عالية جدا للأجسام المضادة لفايروس التهاب القصبات المعدي بعد (14) يوم من ظهور العلامات السريرية، ونتائج (RT-PCR) كانت موجبة لجميع العينات المجموعة (القصبة، الرئة والكلية) في الطور الأول أو الحاد لظهور المرض كما إن نتائج الزرع النسيجي على خلايا أجنة الأفراخ أظهرت تغيرات مرضية على تلك الخلايا بعد حقن الفايروس عليها لأجل عزله.

ولأجل تثبيت النتائج تم إحداث الإصابة التجريبية على (20) طير بعمر (22) يوم فظهرت العلامات السريرية بعد (36) ساعة من إعطاء الفايروس وان نسبة الهلاكات وصلت إلى (33%) في نهاية الإصابة التجريبية وعند دراسة التغيرات النسيجية التي أحدثها الفايروس على (القصبات، الرئة والكلى) لوحظ احتقان واضح مع ارتشاح الخلايا اللمفاوية في تلك الأعضاء.

الكلمات المفتاحية: عزل، فيروس التهاب القصبات الهوائية المعدي، فروج اللحم.