Evaluation of the Immune Response to Live Infectious Bronchitis Disease Vaccines and Their Effect for the Protection against Renal Damage of Layer Chickens in Upper Egypt

AL Hussien[1], M. Dahshan and A.S. Hussien[2]

[1] Department of Poultry Diseases, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511 Egypt.
[2] Department of Virology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt.
ahmadrahini@yahoo.com

Abstract: Infectious bronchitis virus (IBV) is a major highly contagious cause of respiratory infection and poor egg-laying performance in layer chickens in Upper Egypt. Infectious bronchitis virus considered as one of the most common and difficult poultry diseases to control. In this study we make evaluation of the ability of the infectious bronchitis (IB) Ma5 and 4/91 live-attenuated vaccines to protect against kidney damage caused by a Upper Egypt local nephropathogenic IBV strain closely related to Massachusetts (Mass) serotype was isolated from layer chickens farms, field cases showing typical kidney lesions and after serial passages in (SPF) embryonated chicken eggs can isolate these local isolate from layer chickens farms in Upper Egypt and The isolate was serologically identified by Dot-ELISA. The protection parameters considered were gross and microscopic renal pathology, and the use of a polymerase chain reaction to detect IB RNA in kidney tissue. Conclusion: By each parameter, 4/91 alone or the combined program both protected well. But Ma5 vaccine alone provided low protection; a good antibody response and a good level of protection against IBV 4/91 can be achieved by a vaccination program based on live priming with IB (Massachusetts type Ma5) and IB 4/91, followed by vaccination with an inactivated vaccine of the Massachusetts type this will provides. This program will provide antibody titres throughout the life cycle of the hens not only against Massachusetts but also against IBV 4/91. The results confirm the validity of the concept of cross-protection and emphasis the importance of carefully designing vaccination programs to control new variant serotypes under field conditions.

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1. Introduction

Infectious bronchitis (IB) is one of the major viral poultry diseases affecting both layer and broiler chickens causing high economical losses as high mortality, morbidity, decreased egg production and hatchabilities all over the world **(Alexander, 2000).** Many different IBV serotypes and variants are recognized on the basis of antigenic variation, determined by virus neutralization (VN) or molecular analysis of IBV genome. Serotypes of IBV with economic importance such as Massachusetts, which is the serotype most commonly found worldwide. Although control of IBV is primarily through the use of live attenuated vaccines, the disease is difficult to control because different serotypes of the virus do not cross-protect. Therefore, it is necessary to quickly and accurately detect the presence of the virus within an infected poultry flock so that subsequent flocks can be properly vaccinated. It is also important to rapidly differentiate IBV infections from other upper-respiratory diseases like avian influenza, Newcastle disease, infectious laryngotracheitis, and avian mycoplasmosis so that appropriate measures against those diseases can be taken in a timely manner. In Egypt, isolates related to Massachusetts, D3128, D274, D-08880, 4/91 and the novel genotype ; Egypt /Beni-Suef /01 was isolated from different poultry farms **(Abdel-Moneim et al.,2005; Abdel-Moneim et al.,2002; El-Kady,1989 and Sheble et al.,1986)** .The commonly used IBV attenuated vaccine is H120 while the Mass 41 (M41) strain is commonly used in inactivated vaccines. Maternal antibodies can effectively prevent successful vaccination between 10 and 20 days after hatching **(Uenaka, et al., 1998).** Prevention of IBV infections in chicken is based mainly on vaccination as established by **Saif et al., 2003.** Nephritis associated with IBV reported as a major problem in Australia, as the work on IBV associated with nephritis was performed **(Cumming, 1963).** Then the condition has been reported with increased incidence in other countries **(Song et al.,1998).** The renal damage was observed in IB-vaccinated layer flocks, suggesting that the currently used IB vaccination programs may not be providing adequate protection. Studies in Egypt showed that vaccines of the Massachusetts serotype, the most commonly used IB vaccine, protected poorly against challenge with an Egypt/F/03IB isolate and suggested

the need for a homologous vaccine to protect against this challenge **(Abdel-Moneim et al.,2006).** The aim of the present study was to confirm the validity of the concept of cross-protection and determine whether this vaccination protocol protected the kidney against challenge with an IBV capable of inducing nephritis

2. Material and Methods

1-Chickens

Two hundred and fifty one-day-old loghmann chickens were used (El Wadi Company for poultry production, Doki, Giza). The chickens were fed a balanced commercial ration as recommended in the feeding chart provided by the company. These chicks were derived from breeders vaccinated with both live and inactivated IB and NDV vaccines and were used in both virulence test and protection study.

2- Virus and Vaccines

The IBV strains used in this study were the Upper Egypt reference isolate strain isolated in 2011, IBV Ma5 (Massachusetts serotype) and 4/91 live-attenuated vaccines were provided by (Intervet International, co.) were given via oculonasal route in accordance with the recommendations for field application These IBV strains were used as positive control in RT-PCR tests.

3-Embryonated chicken eggs

SPF E.C.E obtained from Nile SPF (Koom Oshiem, Fayoum, Egypt) were used for isolation of the field isolate, serial passages, titration of the isolate and vaccines strains (Ma5) and 4/91, as well as virus re-isolation attempts following challenge in the protection study. Virus isolation was performed in specific pathogen-free (SPF) 9–11-day-old embryonating chicken eggs was isolated from layers flocks suffering from both respiratory and renal distresses from Beni-Seuf,Fayoum and El Menia Governorates in 2011. A kidney homogenate (10% in sterile PBS) and a tracheal scraping suspension were pooled, centrifuged at 500 × g for 10 min. The supernatant fluid was inoculated into chorioallantoic sac of 10- day-old SPF ECE. Allantoic fluid was harvested after 48 h and was used for re-passage into ECE. Ten eggs of the 5th egg passage were incubated till being 20-day-old and examined for typical lesions of IBV (stunting, curling and urates deposition in ureters) as previously described by **Gelb and Jackwood, (1998).**

4-Viral RNA extraction and RT test

Extractions from 20 allantoic fluid and 30 tracheal swabs taken from experimentally infected birds as well as RNA extracted for sensitivity and specificity analysis and for test evaluation was conducted with the MagMax 96 Total RNA isolation kit (Sigma, co) following the manufacturer's suggestions Viral RNA was extracted from 200 µl of allantoic fluid using RNX-plus™ viral RNA extraction kit (Sigma, co.) according to the manufacture procedures. Extracted RNA was dissolved in 20 µl DEPC water. The reverse transcriptation (RT) reaction was performed using ReverAid™ first strand cDNA synthesis kit (Roche, Indianapolis, IN) with oligo (dt) 18 primers.

5-Single RT-PCR

A single RT-PCR test based on detection of M and N genes of IBV was performed using oligonucleotide primers of MIBVPCR-(5'TAAGCTTTCAGTGGCTTGCTAAGTGTGA ACC-3') and IBVPCR-(5'TGGATCCACCGCTACCTTCAAACTTG GGCGG-3'). Using these primers made it possible to distinguish all Corona viruses belonging to group III. These primers amplify a fragment of 1020 bp length using the modified procedure described by **Jackwood et al.,1992.** The PCR reaction mixture containing 2.5 µl of cDNA and 22.5 µl of master mix composed of 2.5 µl 10X buffer, 0.75 µl of 50 mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 mM of each primers, 1.25 units of Taq DNA polymerase and 17 µl DD water in total volume of 25 µl was used. The PCR test was preformed with one initial cycle of 94ºC for 5 min, 52 ºC for 2 min and 72ºC for 2 min, followed by 35 cycles: of 94ºC for 1 min, 52 ºC for 2 min and 72ºC for 2 min with final extension at 72ºC for 15 min of using a master cycler gradient Thermocycler (Eppendorf)

6-Agarose gel electrophoreses

The PCR products were analyzed by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide and visualized in a by UV transilluminator (M-15 UVP co.USA).

7-Analytical sensitivity and specificity of RTPCR

The sensitivity of the RT-PCR assay was determined according to the standard method **(Akbari-Azad et al., 2004).** Briefly, serial dilutions of the Upper Egypt isolate IBV virus in sterile PBS solution ranging from 10^{-1} to 10^{-9} were prepared and each dilution, inoculated into five 9-day-old embryonated chicken eggs. The EID50 titration was determined and all dilutions were examined by the single RT-PCR assays for detection of Massachusetts related serotypes. To evaluate the specificity of the RT-PCR.

8-Hyperimmune serum

Rabbit anti-IBV antiserum against Ma5 and 4/91 vaccines were prepared previously in Intervet company lab and used for detection of IBV antigens in Dot- ELISA test.

9-Dot-ELISA for virus identification

A Dot-ELISA was performed according to **Hawkes et al., 1982.** Briefly, NCM of convenient size was cut, marked with water proof ink for

identification and then soaked for 10 min. in distilled water. NCM was laid on absorbent paper and air dried for 5 min. Three µl of CAM homogenate of the 5th virus passage of Upper Egypt isolate positive control (CAM homogenate 48 h after inoculation of Ma5 vaccine and 4/91 vaccine) and negative control (normal CAM homogenate) samples were applied as small spots on the membrane. The dotted membrane was allowed to air dry for 15 min then blocked for 30 min. in Tris buffer (20 mM Tris base, 500 mM NaCl pH 7.5) containing 0.5% Tween 20 then rinsed for 5 min. in Tris buffer. NCM was then incubated for 1 h with rabbit anti-IBV (prepared previously in Intervet company Lab.) predilluted to 1:10 with diluents buffer (Tris buffer containing 0.05% Tween20). Bound antibodies were detected by incubating NCM for 1 h with goat anti-rabbit peroxidase conjugate (Kikegaard and Perry laboratories. Kpl, U.S.A.) prediluted to 1:500 with diluent buffer. NCM was rinsed three times (10 min. each) with Tris buffer after each step. Finally the membrane was incubated for 15 min. in 60 mm Petri dish containing 20 ml 4-chloro-1-naphthol and hydrogen peroxide substrate working solution. The membrane was rinsed with water to stop the enzymatic reaction. Blue dots denote positive reaction.

10- Virulence test

50 one day-old chickens were used. 40 chickens were infected by intraocular instillation of 10^4 EID_{50}/100 µl of Upper Egypt isolate according to **Meulemans et al.,1987** while other birds were kept as control uninfected group. Clinical signs and gross postmortem lesions as well as mortalities were recorded. Microscopic examinations of both tracheae and kidneys were performed at 5 and 7 days post infection.

11- Challenge test

60 commercial 1-day-old chickens were used to evaluate the protection provided by Ma5 and 4/91 vaccination against challenge with Upper Egypt isolate. Birds were divided into three groups; A_1 (n = 15) A_2 (n = 10), B_1 (n = 15) B_2 (n = 10), C (n = 10). Vaccination was performed at day 1 by eye drop application. Single dose of Ma5 vaccine (Intervet, The Netherlands BV) was used for each bird in groups A_1, A_2 and 4/91 vaccine for B_1, B_2 according to manufacturer's instructions while birds in group C were kept as unvaccinated control. Four weeks post vaccination, chickens in groups A_1 and B_1 were challenged by eye drop with Upper Egypt isolate (10^6 EID_{50} per bird) while birds in group A_2, B_2 were not challenged and kept as vaccinated unchallenged control and the group C kept as unvaccinated control. Tracheae of all birds from all groups were collected 5 days post challenge for virus reisolation attempts and histopathological examination. Tracheal scrapings were emulsified in 2 ml of sterile PBS and centrifuged at 500 × g for 3 min. Virus reisolation attempts were performed by inoculating five, 10-day-old SPF ECE by the supernatant fluid of each sample as described **(Gelb and Jackwood, 1998).** Embryos were examined for typical lesions of IBV. For histopathological examination, tracheae were fixed in formalin, processed routinely for histopathology and stained with haematoxylin and eosin. The trachea from each bird was examined microscopically and assigned lesion scores of 0–3 with 0 = none, 1 = focal, 2 = multifocal, 3 = diffuse. Tracheae were scored for the amount of mucous, loss of cilia, epithelial hyperplasia, necrosis, lymphocyte and heterophil infiltrations as well as the extent of tissue reaction. The scores for each bird were added and the mean score for the birds in each group was calculated **[7].** Kidney samples were also taken 5 days post challenge and examined microscopically for tubular degeneration and inflammation consistent with interstitial nephritis. Focal, multifocal and diffuse were used to assign kidney histopathology.

3. Results and Discussion

In this study, an Egyptian IBV strain; Upper Egypt isolate strain was isolated from a tissue pool of kidney and trachea from unvaccinated layer flock with a history of respiratory and renal disease. The strain produced typical lesions of IBV in inoculated embryos and identified as IBV by Dot-ELISA and RT-PCR. The isolate was found to be devoid of major concomitant viruses; avian influenza virus, Newcastle disease virus, infectious laryngotracheitis virus, reovirus and adenovirus (data not shown).

The most severe clinical response of IBV appears in very young chickens and the severity is declined in older chickens **(Mohamed, 2005 and Toro et al.,1988. T**his fact explains the high mortality rate observed in 1-day-old chickens that experimentally inoculated with Upper Egypt isolate strain compared to mortality pattern in the original layer flock. In the histopathological examination found that. The presence of acute interstitial nephritis on days 5 and 7 post infections indicated that Upper Egypt isolate strain is a nephrogenic IBV. The results of histopathological findings of the renal tubules were matched with the general findings recorded with nephrogenic IBV strains **(Albassam, 1986 and Purcell et al.,1976).** The microscopic findings in tracheal sections appeared similar to those recorded by **Purcell et al., 1976 and Toro et al.,1988** Including: loss of cilia, degenerative changes of the tracheal mucosa, irregular loss of epithelium, desquamation of the sloughed epithelium in the tracheal lumen and lymphocytic infiltration that ranged from focal aggregation to diffuse massive

infiltration. Presence of petechial haemorrhages in larynx and thymus as well as severe congestion of liver, spleen and lungs in birds dead after IBV experimental infection is in agreement with (**Abdel-Moneim et al.,2005 and Cumming, 1963**) who confirmed the presence of IBV viral antigens in such organs. The immune response to IBV vaccination is based on several criteria including: clinical signs, tracheal histological lesion, virus neutralization, virus re-isolation from trachea, In this study we used tracheal histological lesion and virus re-isolation as parameters for tracheal protection. Kidney histopathology was used as indicator of kidney protection. Complete protection is expected upon using closely related vaccine strain as the degree of cross protection among IBV strains, the re-isolation of Upper Egypt isolate strain from the trachea of vaccinated birds and the presence of tracheal and renal microscopic lesions in Ma5 vaccinated birds denote lack of complete protection afforded by Ma5 vaccination. Ma5 is a mild vaccine and it is possible that the challenge virus was too virulent for the level of immunity that the vaccine produced in these young chickens. OR consideration includes that baby chickens are not fully immunocompetent at one-day of age, the time that they were vaccinated for the protection study experiment. However, commercial layer chickens possess maternally derived antibodies, are routinely vaccinated at one-day of age without apparent interference by the maternal derived antibodies in the development of active immunity, at least in the respiratory tract that measured by challenge **(Raggi and Lee, 1965).** Less cross-protection, although the numbers were not statistically significantly different., the kidney damage seen in this experiment was maximum kidney damage was observed in the challenge control chickens was 5 to 7 d.p.c. It is possible that, in the present study, more severe lesions may have been seen if the experiment had continued beyond 10 d.p.c. However, the present results do confirm the previous reports of the severe renal damage that the B1648 strain of IBV can cause **(Meulemans et al., 1987).** Vaccines of the Massachusetts serotype were not effective against challenge with this IBV strain. This finding is confirmed in the present paper. Therefore, this finding is not surprising. Although the Ma5 vaccine used alone did protect against mortality, it provided poor protection against the nephritis caused by this virus. **Abdel-Moneim et al.,2006** suggested that a homologous vaccine is required to control the nephritis caused by this virus. The present results show that this is not necessarily the case. The results of the gross and histological examination of the kidneys and the use of a PCR to detect IB nucleic acid in homogenized kidney and tracheal tissues show that the 4/91 vaccine used alone, and particularly the combined vaccination programme, provided excellent protection against this challenge. This confirms the value of a combined Ma5 and 4/91 vaccination programme in protecting against another manifestation of IB infections. In the present work, Upper Egypt isolate strain known to be capable of inducing nephritis was used. Therefore, it is not known whether similar protection would be achieved against challenge with other nephrotropic IBVs. The PCR technique is used increasingly in the diagnosis of IB infections **(Jackwood et al.,1992 and Meulemans et al., 1987).** Its use in the present paper provides further data on its value, since very good correlation was found between the PCR results and those of the pathological investigation.

Table 1: Protection of chickens following vaccination with IBV Ma5, 4/91 strains and challenged with Upper Egypt isolate strain

Kidney histopathology				*Virus reisolation		challenged	vaccinated	number	Item
Diffuse	Multifocal	Focal	Normal	-ve	+ve				
0	1	8	6	7	8	+	+	15	[a]Group A_1
0	0	0	10	10	0	-	+	10	[b]Group A_2
0	0	3	12	7	5	+	+	15	[c]Group B_1
0	0	0	10	10	0	-	+	10	[d]Group B_2
0	0	0	10	10	0	-	-	10	[e]Group C

a: group A_1 vaccinated by IBV Ma5 vaccine at 1-day of age and challenged by Upper Egypt isolate strain, **b:** group A_2 vaccinated by IBV Ma5 vaccine at 1-day of age and kept unchallenged, **c:** group B_1 vaccinated by IBV 4/91 vaccine at 1-day of age and challenged by Upper Egypt isolate strain, **d:** group B_2 IBV 4/91 vaccine at 1-day of age and kept unchallenged**,e:** group **C** were kept as unvaccinated control. * Virus reisolation 3 days post challenge with Upper Egypt isolate strain in SPF ECE. Unprotected tracheal samples showed positive IBV reisolation while protected tracheal samples showed negative results.

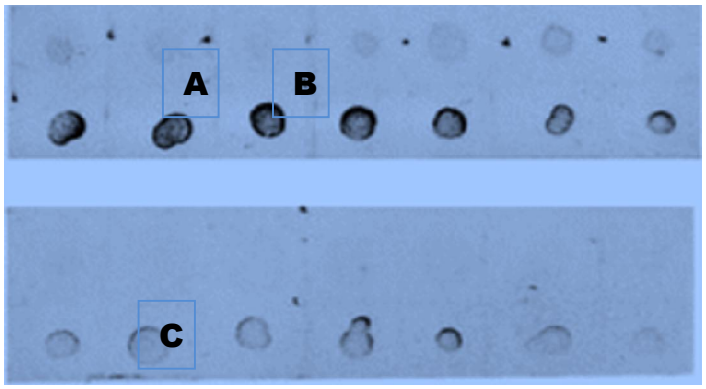


Fig. 1: Dot-ELISA shows positive reaction in tested (chorioallantoic membrane homogenate) and control positive sample. A: control+ve; B: Tested sample; C: Control –ve

RT-PCR test evaluation

A corona virus group III specific single based on conserved regions of M and N genes with 1020 bp was carried out on allantoic fluids as well as the reference antigens of Ma5, 4/91 with MIBVPCR and NIBVPCR primers. Only 20 out of 50 allantoic fluid samples, 30 out 100 tracheal field samples, 8 tracheal swabs from group A_1out of 15 and 5 tracheal swabs from groupB_1 out of 15 showing positive results in single RT-PCR (Fig. 2). The detection limit of the RT-PCR was determined to be approximately equivalent to 10 4 EID50.

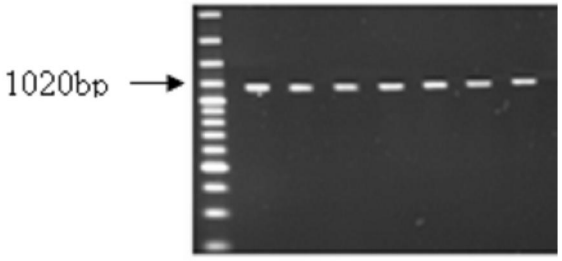
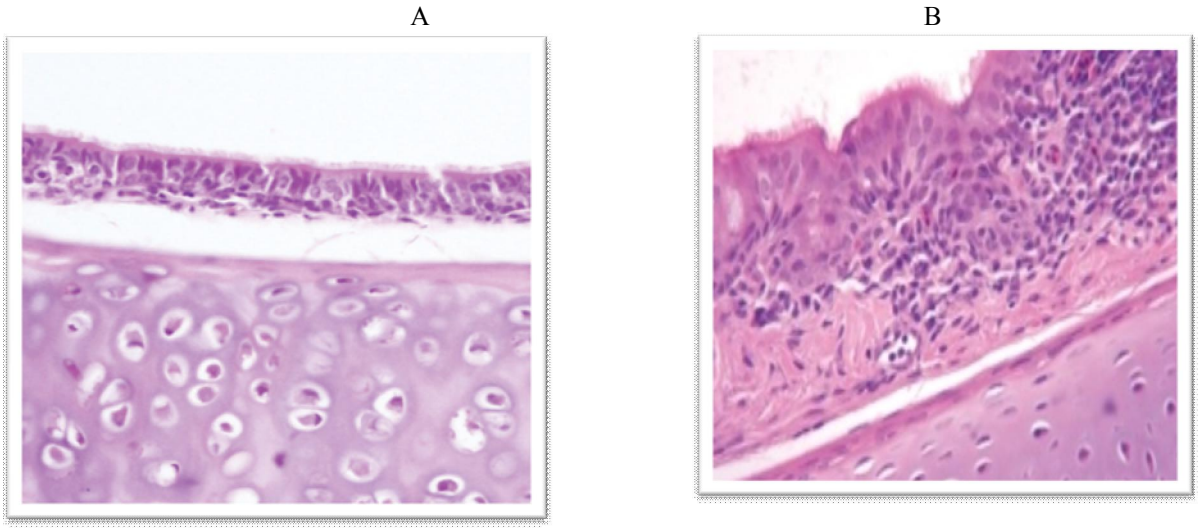
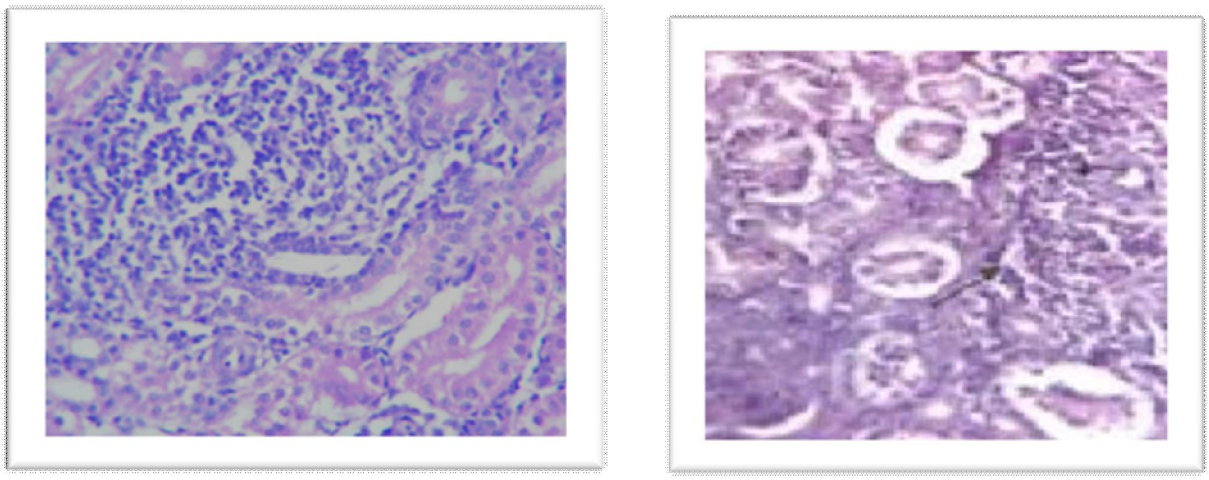


Fig. 2: Agarose gel electrophoresis of IBV specific products amplified by single RTPCR. Lane 1 shows molecular size marker (100 bp plus DNA ladder fermentas); and Lane 2 to 8 show a 1020 bp band related to M and N genes of corona virus group III.



Trachea histopathology following experimental infection of 1-day old chickens with Upper Egypt isolate Trachea stained with H & E: **A:** Trachea of chickens 5 d P.I with Upper Egypt isolate showed hyperplasia, lymphcytic infiltration and oedema (40×). **B:** Trachea of chickens 7 d P.I Upper Egypt isolate showed diffuse lymphocytic aggregation, degeneration of the epithelium mucus, and haemorrhages (20×).

C D



Kidney histopathology following experimental infection of 1-day old chickens with of chickens 5 d P.I with Upper Egypt

c: kidney showed focal lymphocytic aggregation in the interstitial and in the glomeruli, as well as degenerative changes in tubular epithelium (40 ×). d. Kidney of chickens 7 d P.I with Upper Egypt showed massive renal haemorrhages and degeneration renal tubular epithelium (20 ×)

Conclusion

Upper Egypt strain isolate is a nephropathogenic IBV strain closely related to Mass serotype. Vaccination by Ma5 vaccine did not provide satisfactory protection against challenge with Upper Egypt strain isolate. Complete protection of trachea against the Upper Egypt strain isolate and consequently efficient prevention of kidney infection may be a good antibody response and a good level of protection against IBV 4/91 can be achieved by a vaccination program based on live priming with IB (Massachusetts type Ma5) and IB 4/91, followed by vaccination with an inactivated vaccine of the Massachusetts type this will provides. This program will provide antibody titres throughout the life cycle of the hens not only against Massachusetts but also against IBV 4/91. Preparation of live and inactivated vaccines from indigenous isolates should parallel periodic evaluation of cross- protective capabilities of such vaccine(s) versus recently recovered field isolates in order to ensure optimum control of IBV.

Corresponding author
AL Hussien, M. Dahshan
Department of Poultry Diseases, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511 Egypt.
ahmadrahini@yahoo.com

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