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Molecular and histopathological investigation of Mycoplasma *gallisepticum* and *Mycoplasma synoviae* isolates from chickens

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A B S T R A C T

The aim of this work was planned to study the prevalence of mycoplasma organisms in chickens from different localities of Kaluobia, Monofia and Gharbia governorates. In this study a total of 36 farms were examined for prevalence of mycoplasma organisms, these farms were : 3 layer, 20 broilers, 12 balady and one breeder, these farms were located in El Monofia, El Gharbia and El Kaluobia Governorates through application of two methods for diagnosis of *Mycoplasma gallisepticum*(MG) and *Mycoplasma synoviae* (MS). Flocks were examined for the detection of MG and MS infection by isolation and polymerase chain reaction (PCR). In layers 3 flocks were examined for presence of MG, the overall incidence were 14.66% out of the 12% from diseased birds and 2% from apparently healthy one. In broiler from 20 flocks were examined for presence of MG, the overall incidence were 30.38% out of the 33.63% from diseased birds and 12.5 % from apparently healthy. In balady from 12 flocks were examined for presence of MG, the overall incidence were 23.47% out of the 24.54% from diseased birds and 0 % from apparently healthy 4 out of 100 (4%) and 20 out 100 (20 %) for MS and MG respectively in diseased breeder in one flock . *Mycoplasma gallisepticum* field strain was sequenced and compared with the data base on Genbank. The Sequence analysis confirmed the presence of mgc2 virulent gene. The sequenced MG field strain was used in a laboratory experiment to confirm its pathogenicity through studying the clinical signs, body weight and histopathological lesions and minimum inhibitory concentration (MIC) for antibiotics and found that Tiamulin and Doxycycline gave lower concentration. Therefore, identification of Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) using PCR is more accurate and faster than ordinary identification.

Key words: Chickens, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, PCR, Sequencing.

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1. INTRODUCTION

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are infectious agents of chronic respiratory disease in chickens (Feberwee, 2005).

Mycoplasma synoviae infection most frequently occur as subclinical upper respiratory disease that may become systemic and result in infectious synovitis that in an

acute or chronic infections of chicken and turkeys involving primary the synovial membrane of joint and tendon sheaths (Kleven, 1997).

The presence of MG and MS results in severe direct and indirect losses to the poultry industry (Buim *et al.*, 2009).The losses include decreased hatchability, drop in egg production, poor quality chicks, reduced growth rate, in addition to increasing costs with disease eradication procedures monitoring and control programs (Yilmaz *et al*., 2011).

The serological tests are poorly specific, but PCR has proved higher sensitivity and faster (Sprygin *et al*., 2010).

Control of pathogenic avian mycoplasma occurred through three ways, maintaining flocks free from infection by strict biosecurity, medication and vaccination. The periodical using of some anti-mycoplasma drugs lead to resistance to these drugs so it must be a periodically checked using sensitivity test.

Therefore the aim of this work was planned to 1) Study the prevalence of mycoplasma organisms in different type of breeding chickens from El- Monofia, El- Gharbia and El- Kaluobia Governorates for diagnosis of MG and MS using isolation and PCR, 2) Determine minimum inhibitory concentration (MIC) of the isolated Mycoplasma strain against some anti-mycoplasma drugs to reach the suitable dose of drugs, and 3) Detect the virulence of the isolated strain.

2. MATERIALS AND METHODS

2.1.Samples:

Five hundred and fifty samples were collected from three different Governorates (El Monofia, El Gharbia and El Kaluobia). The ages, types of breeds were shown in table (1). The total number was 550 samples. The number of samples was 353 from El-Monofia, 125 from El-Kaluobia and 72 from El-Gharbia i.e.

Table (1) Historical data sheet of examined chickens flocks

Breed		Age (days)	Live brides			Slaughtered birds	Governorates (1. El Monofia) (2. El Kaluobia) (3. El Gharbia)
			Signs		Tracheal swaps	Tissue samples (air sac, lung and trachea)	
			Positive	Negative			
Broiler	Avian	1-35	35	10	30	15	1 + 2
	Ross	1-37	62	20	50	32	1 + 2 +3
	Cobb	22-40	123	-	90	33	1+ 2
	Hubbard	1	-	10	10	-	1
Balady		20-180	70	5	40	35	1 + 3
Saso		4-35	40	-	-	40	1
Layer (hysex)		45-118	50	25	75	-	1
Breeder		280	100	-	95	5	1
Total		-	480	70	390	160	

2.2.Isolation and biochemical Identification:

Samples were cultured by inoculation on Frey's broth media (Frey et al., 1968) then plated on PPLO agar medium (Sabry and Ahmed, 1975) and incubated at 37 ℃ for 3-7 days. Digitonin sensitivity was done to differentiate between Mycoplasma and Acloeplasma (Freundt, 1973). Biochemical identification was done as described by (Erno and Stipkovits, 1973). Film & Spot formation test according to (Fabricant and Freundt, 1967).

2.3. Molecular detection of mycoplasma

2.3.1. DNA Extraction: The DNA was extracted with DNA extraction kit (QIAamp DNA Mini Kit Qiagen Germany, Cat. No. 51304). DNA was kept at -20 ˚C till used for PCR.

2.3.2. Primer

The primers used in the study for detection of MG and MS using 16SrRNA gene (OIE. 2008) , mgc2 virulent gene of MG (Garcia et al.,2005) and finally vlhA gene for MS (Zhao and Yamamoto 1995) Table (1).

2.3.3. Polymerase Chain Reaction procedure:

All the final 50-µl reaction volumes contained 25 of master mix, 3 µl of DNA, 1 µl from each of the two primers (each gene separate), and 20 µl DNase, RNase free water. Amplification was performed in a programmable thermal cycler (Bio-Rad S1000) with 40 cycles of: denaturation at 94°C for 30 Sec, annealing at 58° C for 30 Sec and extension at 72°C for 1 min then final extension at 72°C for 5min for MG and MS 16SrRNA gene (OIE). Initial denaturation 95 for 3 min. followed by 35 cycles of: denaturation at 94°C for 30 Sec, annealing at 58° C for 40 Sec and extension at 72°C for 1 min then final extension at 72°C for 5min for MG mgc2 gene (Garcia et al., 2005). An initial denaturation step (95 ºC for 10 min), followed by 35 cycles of denaturation (95°C for 1 min), annealing (55°C for 2 min), extension (72ºC for 2 min) and A final extension step at 72°C for 10 min for MS *vlhA* gene (Zhao and Yamamoto 1993b). Agarose gel (1.5%) electrophoresis, stained with ethidium bromide, and visualized with an ultraviolet transilluminator then photographed.

2.3.4. Sequencing of purified PCR product of mycoplasma isolates: It was done by Macrogene Company, South Korea.

Sequence analysis: BioEdit sequence alignment editor (Hall, 1999) and MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW (Thompson et al., 1994). Sequence divergence and identity percent were calculated by MegAlign

2.4. Minimal inhibitory concentration (MIC): eight antimicrobials were used in the present study (Doxycycline 20% (Primavet), Ciprofloxacin 10% (Agrovetica), Tylosin 20% (Adwia), Enrofloxacin 10% (El Nasr), Gentamicin 10% (Primavet), Linco-spectin (5% Lincomycin1% Spectinomycin), Oxytetracycline (3%) (Deltafarma), Tiamulin 45% (Novartis)).

Sterile stock solution containing 128 ug/ml were prepared from each in distilled water. They were stored at 4 ºC but used in the day of preparation. The test was done as described by Hannan, 2000.

2.4.1. Pathogenicity experiment:

This experiment was done to determine the pathogenicity of the isolated MG in chickens and efficacy of some antibiotics which gave good results by MIC as tiamulin and doxycycline and the role of prophylactic treatment in prevention of infection. A total number of 25 one day old Ross chicks (broiler) were obtained from El Kholy company, which were negative for mycoplasma as judged by failure to detect the organism by PCR. These chicks were divided to 5 groups: Group 1: no infection and no treatment, Group 2: treated with tiamulin after infection, Group 3: treated with doxycycline after infection, Group4: treated with tiamulin before infection, and Group5: infected by MG and not treated.

2.5. Histopathology

Tissues collected for histopathological examination were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 to 6 m and stained with hematoxylin and eosin.

3. RESULTS

3.1. Clinical signs

A summary of the clinical signs on the samples birds is demonstrated in table (1)

Table (1) Results of clinical signs and body weight

Group	Infection and treatment	Clinical signs	Body weight after 6 weeks / bird/grams					
			1	2	3	4	5	mean
1	No infection and No treatment	-ve	2000	1960	1890	1880	1980	1942
2	Treated with Tiamulin after infection of MG	-ve	1900	1940	1890	1880	1980	1918
3	Treated with doxycycline after infection of MG	-ve	1900	1970	1920	1880	1900	1932
4	Treated with Tiamulin before infection of MG	+ve	1800	1810	1790	1780	1820	1800
5	Infection by MG No treatment	+ve	1600	1720	1740	1750	1720	1706
6	Infection by MS No treatment	+ve	1700	1750	1700	1720	1680	1710

3.1. Isolation of mycoplasma.

The recovery rate of MG from layer breed chickens were 11 out from 75 (14.6 %), broiler breed was79 out from 260 (30.3%), balady was 27out from 115 (23.47%) and breeder was 20 out from 100 (20%) as MG and4 out from100 (4%) as MS. Nucleotide sequencing (Table 3).

The isolated strains were glucose positive, arginine negative and film & spot negative for MG and film & spot positive for MS (Table 4). Percent Identity was demonstrated in table 5. MIC for MG and MS were presented table (6 & 7). The most effective antibiotics were Tiamulin and Doxycycline gave MIC.

3.2. Molecular detection of mycoplasma isolates from chicken

Agarose gel electrophoresis of PCR product from *Mycoplasma gallisepticum and synoviae* is demonstrated in fig. (1-5).

3.3. Histopathology

Air sacs, trachea, lungs and bronchioles examined for histopathology (Fig. 6-10) and the results illustrated in table (8).

4. DISCUSION

Mycoplasma Gallisepticum colonizes its host mainly via the mucosal surfaces of the respiratory tract, causing air sacculitis within a few days (Much et al 2012), and disseminates throughout the body. This systemic infection is reflected by the high rate of MG re-isolation from inner organs such as the liver, heart, spleen, or kidney (Much et al 2012) and by its detection inside and at the surface of red blood cells of experimentally infected birds (Vogl et al 2008).

The incidence of infection in layer was 14.66%. These results agreed with El-Shater (1986), who isolated MG from 16.7% of the examined birds. Also, these results agreed with Abd El-Gwad., (2005), who isolated MG from Balady breed (45w) (18.33%) in Kaluobia Governorate.

Table (3) Nucleotide sequences and anticipated sizes of PCR products for the *M. gallisepticum and Mycoplasma synoviae* gene-specific oligonucleotide

Primer	Oligonucleotide sequence (5'-3')	Size of amplified Product
16srrna MGF 16srrna MG R	GAGCTAATCTGTAAAGTTGGTC GCTTCCTTGCGGTTAGCAAC	185 bp
16srrna MS F 16srrna MS R	GAGAAGCAAAATAGTGATATCA CAGTCGTCTCCGAAGTTAACAA	213 bp
MG-mgc2 F MG-mgc2-R	CGCAATTTGGTCCTAATCCCCAACA TAAACCCACCTCCAGCTTTATTTCC	300 bp
MS-vlha MS-vlha	ATTAGCAGCTAGTGCAGTGGCC AGTAACCGATCCGCTTAATGC	1100 bp

Table (4) Isolation and biochemical results of isolated of Mycoplasma strains

Breed	No. Of sample	No +ve	%	Digitonin		Glucose		Arginine		Film & spot	
				+ve	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*
Layer	75	11	14.6	11	-	11	-	-	11	-	-
Broiler	260	79	30.3	79	-	79	-	-	79	-	-
Balady	115	27	23.5	27	-	27	-	-	27	-	-
Breeder	100	30	30	30	-	30	-	-	30	4	26

Table (5) Percent Identity Matrix - created by Clustal 2.1

1: Rab108	100.00	98.67	98.67	98.67	100.00	99.67
2: MGS1210	98.67	100.00	100.00	100.00	98.68	98.34
3: MGS1167	98.67	100.00	100.00	100.00	98.68	98.34
4: MGS19B	98.67	100.00	100.00	100.00	98.68	98.34
5: NouhC15mg C2	100.00	98.68	98.68	98.68	100.00	99.67
6: Eid1MGTKEG014	99.67	98.34	98.34	98.34	99.67	100.00

Nouh C15 mg C2100%identfy with Rab108 strain, 99.67 with Eid1MGTKEG014 and 98.67with: MGS1210, MGS1167 and MGS19B

Table (6) The results of Minimum inhibitory concentration of *Mycoplasma gallisepticum*

antibiotic	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	mean	S6 Ref. strain
Doxycycline	0.031	0.031	0.062	0.031	0.032	0.031	0.031
Ciprofloxacin	0.5	1	1	2	0.5	1	8
Enrofloxacin	0.062	0.31	0.125	0.062	0.062	0.062	0.125
Gentamicin	0.125	0.65	0.125	0.062	0.125	0.125	0.125
Linco-spectin	0.5	1	0.25	0.5	0.5	0.5	1
Oxytetracycline	2	4	1	2	2	2	resistant
Tylosin	1	2	0.5	1	1	1	2
Tiamulin	0.031	0.031	0.031	0.031	0.031	0.031	0.031

Table (7) The result of Minimum inhibitory concentration of Mycoplasma synoviae isolated from examined governorates

Antibiotic	Isolate 1	Isolate 2	Isolate 3	Isolate 4	mean	WVU reference strain
Doxycycline	0.031	0.031	0.031	0.031	0.031	0.031
Ciprofloxacin	1	2	2	1	1	4
Enrofloxacin	0.062	0.062	0.062	0.062	0.062	0.125
Gentamicin	0.062	0.062	0.031	0.062	0.062	0.062
Lincospectine	0.125	0.125	0.250	0.125	0.125	0.250
Oxytetracyclin	1	2	2	2	1	2
Tylosin	0.5	0.5	1	0.5	0.5	1
Tiamulin	0.031	0.031	0.062	0.062	0.031	0.031

Broiler flocks reviled recovery rates 30.3%, Sokkar *et al.*, (1986) recovered MG from trachea, sinuses and air sacs from 50 chickens of different ages by tracheal swabs with 24% In balady breeds 27 out 115 samples were positive for MG with percentage of 23.47 %, also, these results agreed with Abd El-Gwad (2005), who isolated MG from Balady breed (45w) (18.33%) in Kaluobia Governorate.

Timms (1967) stated that in spite of difficulties encountered in the isolation and identification of avian Mycoplasma during

routine diagnostic work, he was able to recover 218 Mycoplasma isolates from a total of 551 respiratory and synovial specimens using different methods

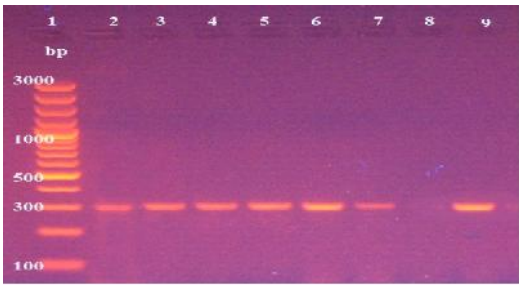

Fig. (1) Agarose gel electrophoresis of PCR product from *Mycoplasma gallisepticum* reference strains and some field isolates using mgc2 gene primer (Garcia et al., 2005). Lane 1: 100bp DNA ladder (Pharmacia). Lane 2-7 *M. gallisepticum* field isolates. Lane 8: control Negative. *Lane 9: M. gallisepticum* strain (control positive).

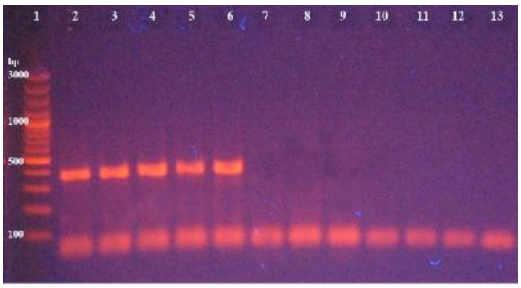

Fig. (3) Agarose gel electrophoresis of PCR product from *Mycoplasma synoviae* reference strains and some field isolates using *vlhA* gene. Lane 1: 100bp DNA ladder (Pharmacia). Lane 2: control positive M. *synoviae*. Lane 3: control Negative *M. synoviae*. Lane 4-10: *M. synoviae* field isolates

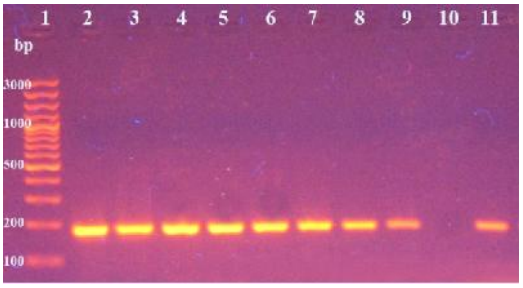

Fig. (2) Agarose gel electrophoresis of PCR product from *Mycoplasma gallisepticum* reference strains and some field isolates using OIE primer.(185 bp). Lane 1: 100bp DNA ladder (Pharmacia). Lane 2-9: *M. gallisepticum* field isolates. Lane 10*:* control Negative. Lane 11: *M. gallisepticum* strain (control positive).

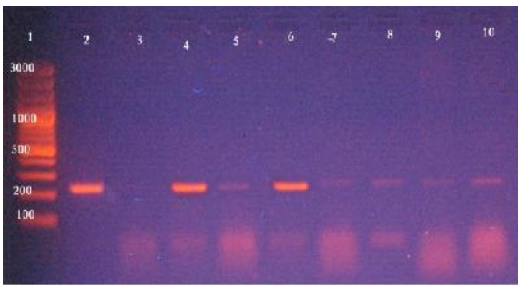

Fig (4) Agarose gel electrophoresis of PCR product from *Mycoplasma synoviae* reference strains and some field isolates using OIE primer (215 bp). Lane 1: 100bp DNA ladder (Pharmacia). Lane 2: control positive M. *synoviae*. Lane 3: control Negative *M. synoviae*. Lane 4-10: *M. synoviae* field isolates

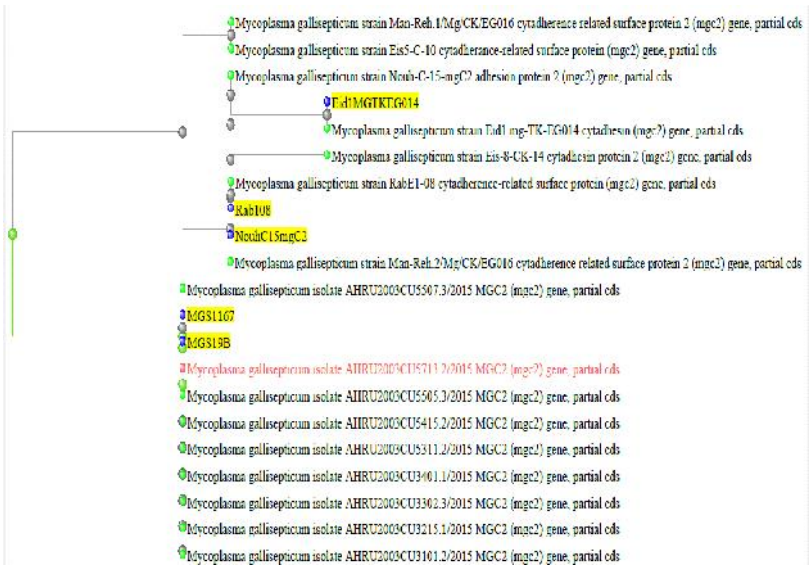

Fig. (5) Sequence results and phylogenetic tree

PCR is a rapid, sensitive and accurate test which depends on the detection of DNA even in non-viable mycoplasma. In the present work, PCR assay was used for confirmation

the identified mycoplasma strains. Common genes were detected in all MG strains via 16sr RNA, mgc2 genes for MG; also, vlhA gene was detected in MS strains. Our results agreed with Garcia et al., (2005), who compared and evaluate various PCR methods for detection of MG infection in chickens. They found that cydtadesin membrane surface protein (mgc2) is the best to be used in PCR due to its importance as a virulence factor which provide mycoplasma with resistance to host defense. Also, Zhao and Yamamoto, (1993) evaluated species specific PCR assay for the detection of vlhA virulent gene *of* MS; they detected a PCR fragment at 1100 bp. In the current study, a MG field isolate (mgc2 gene) was sequenced, analyzed and compared with data base on GenBank, the results was a new strain named Nouh-C15-mgc2. A laboratory experiment was done to study the pathogenicity of MG field strain (Nouh-C15-mgc2) and the efficacy of treatment with Tiamulin and doxycycline (before and after infection). Histopathology of internal organs of infected birds showed pathological lesions, indicating the virulence of the infected MG strain. Tiamulin was affective when given as prophylactic before infection, while doxycycline was effective for treatment of Mycoplasma infection Eissa et al. (2000) concluded that treatment of chickens infected with MG using Tiamulin resulted in increase of body weight and decrease of pathological lesions of internal organs. Also, they mentioned that five birds out of ten by PCR were positive at the 2nd and 3rd week post treatment.

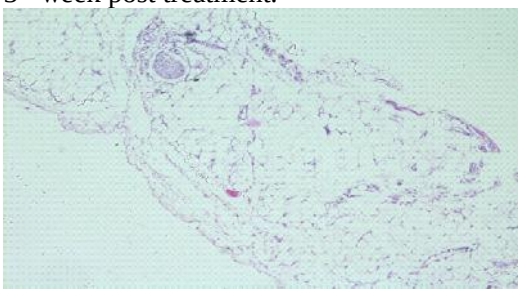

Fig. (6) Air sac of group 1 (No infection, No treatment) showing edema and mild cellular infiltration.H&E.X10

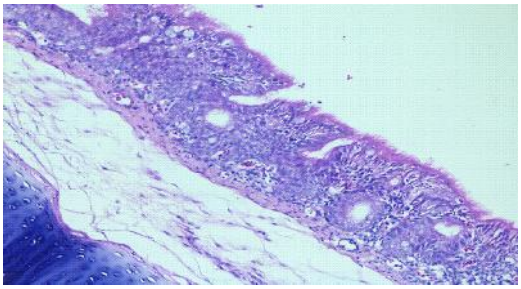

Fig. (7) Trachea of group 2 (Treated with Tiamulin after infection of MG) showing epithelial hyperplasia with vascular degeneration change .H&E X 200

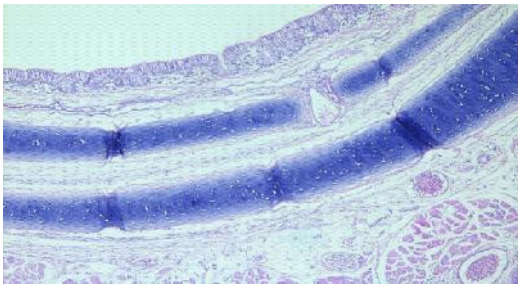

Fig. (8) Trachea of group 3 Treated with doxycycline after infection of MG) showing activation of goblet cells with pocket formation. H&E X 400

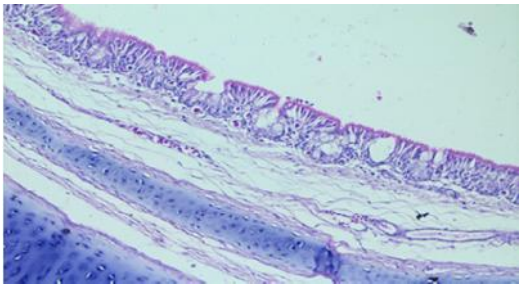

Fig. (9) Trachea of group 4(Treated with Tiamulin before infection of MG showing pronounced activation of goblet cells. H&E.

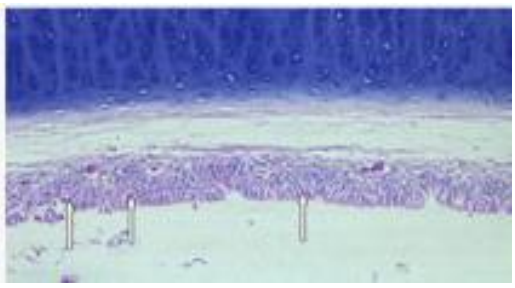

Fig. (10) Trachea of group 5 showing marked epithelial hyperplasia. H&E X 200

Table (8) Results of histopathology

Organ	lesion	Gr.1	Gr. 2	Gr.3	Gr.4	Gr.5
Air sac	▪ Infiltration by inflammatory cell	+|	-	-	-	-
	▪ Thickening	-	++	++	-	-
Lung	▪ Dilatation of pulmonary blood vessels	-	+++	-	-	-
	▪ Emphysema	-	+++	+	++	+++
	Pulmonary tissue					
	▪ Proliferation of inflammatory cell		+++	-	-	
	▪ Replacement by structure less mass	-	++	-	-	-
	▪ Proliferation of fibrous tissue	-	-	++	-	+++
	▪ Congestion of blood vessels	-	++	+	++	+++
	▪ Hemorrhage	-	++	+	++	+++
Trachea	Mucosa:					
	▪ Epithelial Hyperplasia	-	+	-	++	++
	▪ Deciliation	-	+++	-	-	-
	▪ Activation of goblet cell	-	++	++	++	++
	▪ Cystic formation-	-	++	-	-	++
	Submucosa and lamina propria					
	▪ Congestion of blood vessels	-	+++	-	+	++
	▪ Proliferation of inflammatory cell	-	+++	+	-	-
	▪ Thickening	-	++	+	-	++
	Muscular layer					
	▪ Edema of muscular layer	-	++	+	-	++
Bronchiole	▪ Epithelial hyperplasia	-	+++	-	-	-
	▪ Proliferation of inflammatory cell	-	+++	-	-	++

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