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Molecular and histopathological investigation of *Mycoplsma gallisepticum* Isolates From Chickens

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Abstract

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 the prevalence of mycoplasma organisms, these farms were: 3 layer, 20 broilers,12 balady and one breeder, 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 detection of *MG*; 12% from diseased birds and 2% from apparently healthy one. In broiler, from 20 flocks were examined for presence of *MG*, the percentage was 33.63% from diseased bird and12.5% from apparently healthy. Twelve flocks of balady breed were examined for detection of mycoplasma infection, mycoplasma could be detected in 24.54% of diseased birds, while apparently healthy birds gave negative results. On the other hand one flock of breeder was positive for *Mycoplasma synoviae* (4%) and 20% for *MG*. *Mycoplasma gallisepticum* field strain was sequenced and compared with the data base on Genbank. The Sequence analysis confirmed the presence of *mgc*2 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

Keywords*: Mycoplasma gallisepticum, PCR, Chickens, Sequencing*.

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, egg production, poor quality chicks, reduced growth rate, in addition to increase 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 through three ways: maintaining flocks free from infection by strict biosecurity, medication and vaccination and 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: Study the prevalence of mycoplasma organisms in different type of breeding chickens in Menofia, Gharbia and Kaluobia Governorates for diagnosis of MG and MS using isolation

and PCR. Determination of minimum inhibitory concentration (MIC) of the Mycoplasma strains against some anti-mycoplasmal drugs to reach the suitable dose of drugs. And detection the virulence of the isolated strain by laboratory experiment.

Materials and Methods

Samples*:* Five hundred and fifty samples were collected from three different Governorates (Menofia, Gharbia and Kaluobia). The total number was 550 (353 from Menofia, 125 Kaluobia and 72 from Gharbia). The ages, types of breeds were shown in table (1).

Table (1). Historical data sheet of examined chickens flocks

Breed	Age	Living birds			Slaughtered birds
		Diseased	Apparent Healthy	Tracheal swabs	Tissue samples
Avian	1-35 day	35	10	30	15
Ross	1-37day	62	20	50	32
Cobb	22-40day	123	-	90	33
Hubbard	1day		10	10	-
Balady	20-180day	70	5	40	35
Saso	4-35 day	40	-	-	40
Layer (Hysex)	45-118 day	50	25	75	-
Breeder	280 day	100	-	95	5
Total		**480**	**70**	**390**	**160**

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°C for 3-7 days. Digitonin sensitivity was done to differentiate between Mycoplasma and Acloeplasma (**Freundt *et al.*, 1973**). Biochemical identification was done as described previously (**Erno and Stipkovits, 1973**). Film & Spot formation test conducted as described before (**Fabricant and Freundt 1967**).

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.

Primer selection: The primers used in the study for detection of *MG* and *MS* using *16S*rRNA gene (**OIE, 2008**), *mgc*2 virulent gene of *MG* (**Garcia *et al.*, 2005**) and finally *vlh*A gene for *MS* (**Hammond *et al.*, 209**) Table (2).

Table (2). 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 (bp)	References
16SrRNA MGF **16SrRNA MG R**	GAGCTAATCTGTAAAGTTGGTC GCTTCCTTGCGGTTAGCAAC	185	**OIE, (2008)**
16SrRNA MS F **16SrRNA MS R**	GAGAAGCAAAATAGTGATATCA CAGTCGTCTCCGAAGTTAACAA	213	**OIE, (2008)**
MG-mgc2 F **MG-mgc2-R**	CGCAATTTGGTCCTAATCCCCAACA TAAACCCACCTCCAGCTTTATTTCC	300	**Garcia, (2005)**
MS-vlhA **MS-vlhA**	ATTAGCAGCTAGTGCAGTGGCC AG- TAACCGATCCGCTTAATGC	350-400	**Hammond *et al*., (2009)**

Polymerase chain reaction amplification and cycle protocols: 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) Table 3

Table (3). Protocol for amplification condition for PCR

Gene	Cycle condition				Cycle no.	Final Extension Temp./ min.
	Initial denaturation Temp./min.	Denaturation Temp./sec	Annealing Temp./sec	Extension Temp./sec		
MG _MS 16SrRNA	-	94 °C /30	58°C /30	72°C /60	40	72°C / 5
MG *mgc2*	-	94°C/30	58° C/40	72°C/60	35	72°C/5
MS *vlhA*	95 °C/10	95°C/60	55°C/120	72°C/120	35	72°C/10

The PCR products were electrophoresed on agarose gel (1.5%), stained with ethidium bromide (1µl/gel), and visualized with an ultraviolet transilluminator then photographed.

Sequencing of purified PCR product *of mycoplasma* isolate: The amplified products were purified using Gene JET PCR purification kit (USA) and were sequenced by Sigma Company, Egypt. The strain was sequenced in both directions. Sequences were analyzed and aligned by Clustal W (**Thompson *et al.,* 1994**). The strain was recorded in genbank under name *Mycoplasma gallisepticum* strain Nouh-C-*mgc*2 adhesion protein2 *mgc*2) gene, partial cods.

Minimal inhibitory concentration (MIC): Eight antimicrobials were used in the present study. Sterile stock solutions (containing 128 ug/ml) were prepared from each antimicrobial in distilled water. They were used in the day of preparation. The test was done as described by **(Hannan, 2000)**

Experiment: This experiment was done to determine the pathogenicity of 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 twenty five, 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 doxycyclin after

infection, Group4: treated with tiamulin before infection, and Group5: infected by MG and not treated. The chicken groups were intratracheally inoculated with 0.2 x 10^7 ml inoculums of *Mycoplasma gallisepticum* field isolate per bird at 3 weeks old. The birds were observed daily for any respiratory signs or dead cases. The dose of treated antibiotic was 20 mg/kg of tiamulin and 20 mg/kg of doxycycline. A bird from each group inclding the control was selected weekly at the end of 1st, 2nd, 3rd, 4th, and 5th week post-inoculation, slaughtered and examined macroscopically for any lesions in the internal organs especially for collibacillosis and or CRD

Histopathology (Bancreoft and Gamble 2008): Tissues collected for histopathological examination (specimens from lung, tracheas and air sacs) were collected weekly after necropsy and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 to 6 µ m and stained with hematoxylin and eosin (HE) then examined microscopically for histopathology.

Results

Isolation of *mycoplasma:* The recovery rate of Mycoplasma from layer breed chickens were 14.6 %, broiler breed was 30.3%, balady was 23.47% and breeder was 30%. The isolated strains were glucose positive, arginine negative and film & spot negative only one isolate was film and sport positive. Table (4)

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*
Layer	75	11	14.6	11	11	-	-
Broiler	260	79	30.3	79	79	-	-
Balady	115	27	23.5	27	27	-	-
Breeder	100	30	30	30	30	-	1

Polymerase chain reaction results (PCR): The PCR was applied on Mycoplasma isolates recovered from three Governorates. Detection of MG and MS using *16sr*RNA gene (**OIE, 208)** was done for screening the presence of Mycoplasma which gave positive results for all isolates then a primer set of *mgc*2 gene encoding a cytadherence related surface role in the virulence of MG was done. PCR results showed that *mgc2* gene was detected in all examined isolates and gave a characteristic band at 300 bp. Detection of MS using *vlh*A gene gave a characteristic band at 400 bp (Figs 1-4).

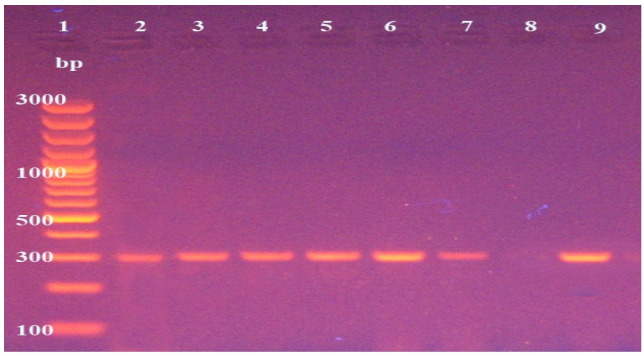

Fig. (1): Agarose gel electrophoresis of PCR product from *Mycoplasma gallisepticum* reference strains and some field isolates using mgc2 gene primer **(Garcia *et al.*, 2005).**
Lane 1: 100bp DNA ladder (Pharmacia).
Lane 2-7 *M. gallisepticum* field isolates**.**
Lane 8: control Negative**.**
Lane 9: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

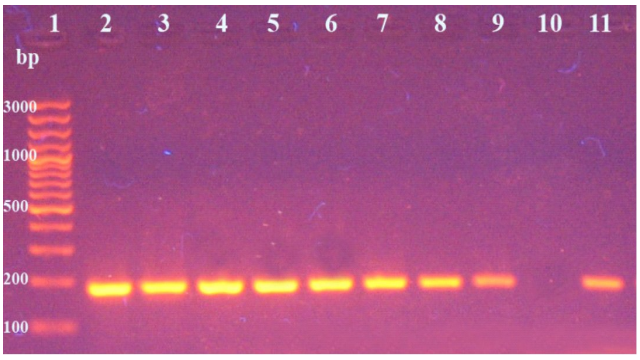

Fig. (2): Agarose gel electrophoresis of PCR product from *Mycoplasma gallisepticum* reference strains and some field isolates using *16sr*RNA gene. (185 bp)
Lane 1: 100bp DNA ladder (Pharmacia).
Lane 2-9: *M. gallisepticum* field isolates.
Lane 10: control Negative.
Lane 11: *M. gallisepticum* strain (control positive).

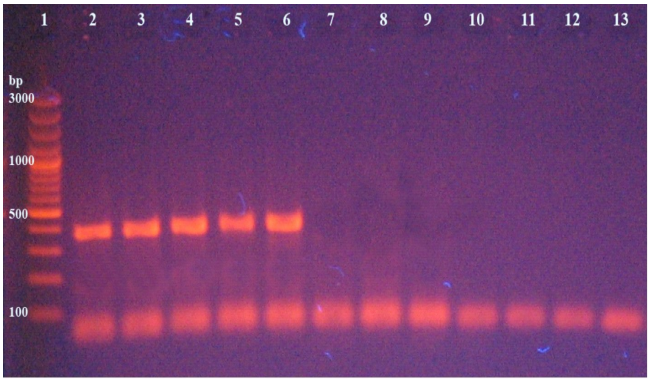

Fig. (3): Agarose gel electrophoresis of PCR product from *Mycoplasma synoviae* reference strains and some field isolates using *vlhA* gene
Lane 1: 100bp DNA ladder (Pharmacia).
Lane 2: *control positive M. synoviae*
Lane 3-9: *M. synoviae* field isolates
Lane 10: *control Negative M. synoviae*

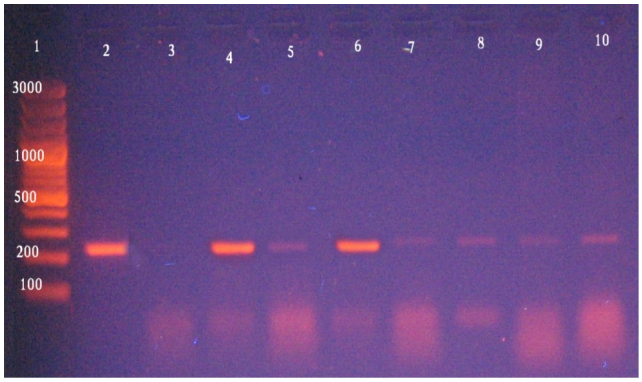

Fig. (4): Agarose gel electrophoresis of PCR product from *Mycoplasma synoviae* reference strains and some field isolates using *16sr*RNA gene (215 bp)
Lane 1: 100bp DNA ladder (Pharmacia).
Lane 2: control positive M. *synoviae*
Lane 3: control Negative *M. synoviae*
Lane 4-10: *M. synoviae* field isolates

Phylogenetic analysis results*:*
Nucleotide phylogenetic tree of *mgc2* showed that our Egyptian MG isolate was located in the same group with: Rab1-08, Eid1 MGT-KEG014, and Eis 5-C-10 and Eis 7 (fig.5). from the identity matrix using Clustal we found that Nouh C15 mgc2 100% identity with Rab1- 08 strain, 99.67 with Eid1 MGT-KEG014 and 98.67 with MG S1210 (Fig.5 and Table 5).

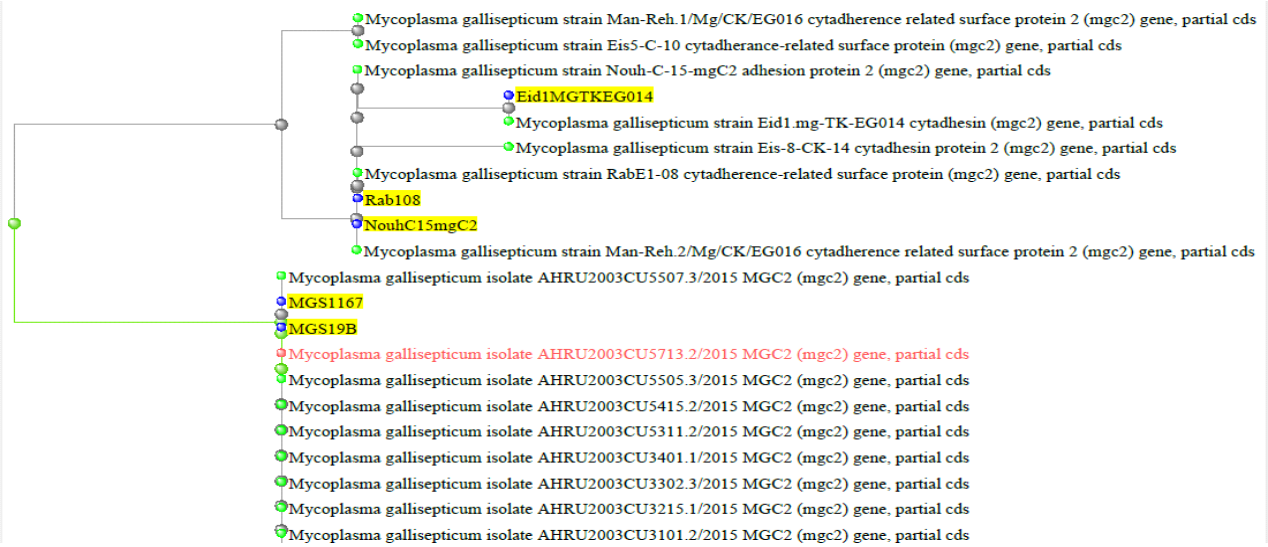

Fig. (5): Phylogentic tree of nucleotide sequence of *M. galllisepticum* (*mgc*2 gene)

Table (5). Percent of identity of nucleotide sequence of *M. galllisepticum mgc*2 gene

	Rab1-08	**MGS1210**	**MGS1167**	**MGS19B**	**Nouh-C-15-*mgc*2**	**Eid1-GTKEG01**
Rab1-08	100.0	98.67	98.67	98.67	100.0	99.67
MGS1210	98.67	100.0	100.0	100.0	98.68	98.34
MGS1167	98.67	100.0	100.0	100.0	98.68	98.34
MGS19B	98.67	100.0	100.0	100.0	98.68	98.34
Nouh-C-15-*mgc*2	100.0	98.68	99.68	98.68	100.0	99.67
Eid1-MGTKEG01	99.67	98.34	98.34	98.34	99.67	100.0

Results of Minimal inhibitory concentration: All isolates of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were sensitive to Doxycycline and tiamulin and resistant to oxytetracycline

Table (6). Results of Minimum inhibitory concentration of MIC of MG and MS

Antibiotic	**Isolates of *M. gallisepticum***							**Isolates of MS**	
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**	**mean**	**S6 Ref. strain**	**Isolate1**	**reference strain**
Doxycycline	0.031	0.031	0.062	0.031	0.031	0.031	0.031	0.031	0.031
Ciprofloxacin	0.5	1	1	2	0.5	1	8	1	4
Enrofloxacin	0.062	0.31	0.125	0.062	0.062	0.062	0.125	0.062	0.125
Gentamicin	0.125	0.65	0.125	0.062	0.125	0.125	0.125	0.062	0.062
Lincospectine	0.5	1	0.25	0.5	0.5	0.5	1	0.125	0.250
Oxytetracyclin	2	4	1	2	2	2	Resis.	1	2
Tylosin	1	2	0.5	1	1	1	2	0.5	1
Tiamulin	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031

Results of Experiment*:* An increase in body weight was noticed in all infected groups compared with positive control group during the whole period of the experiment, but without significant difference, Table (7)

Table (7). Results of clinical signs and body weight

Groups	Infection and treatment	Clinical signs	Body weight after 5 weeks/ bird/grams					Mean
			1	2	3	4	5	
1	No-infection 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

Histopathology Results: Air sacs, trachea, and lungs were examined for histopathology. The results revealed that **air sac:** thickening in group2 and 3**,** while in **lung:** dilation of pulmonary blood vessels, emphysema and proliferation of inflammatory cells in group2. Groups 4 and 5 showed emphysema, proliferation of fibrous tissue, congestion of blood vessels and hemorrhage. Group 3 showed proliferation of fibrous tissue. In **trachea:** group 2, showed deciliation, proliferation of inflammatory cells, activation of goblet cells, congestion of blood cells and thickening.

Group 3: showed actuation of goblet cells and weak proliferation of inflammatory cells. Group 4: showed epithelial hyperplasia, actination of goblet cells and less congestion of blood vessels. Group 5: showed epithelial hyperplasia, activation of goblet cells, cystic formation, and congestion of blood vessels (Figs. 6-9 and Table 8).

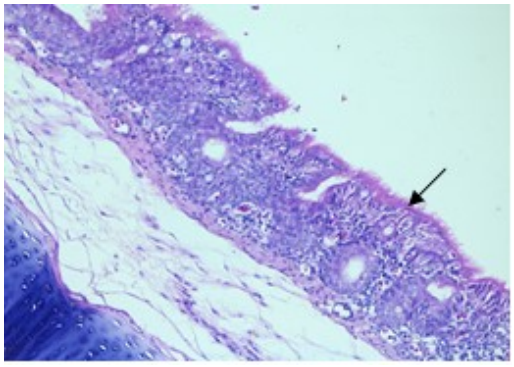

Fig. (6): Trachea of group 2 (Treated with tiamulin after infection of MG) showing epithelial hyperplasia with vascular degeneration change . H&E X 20

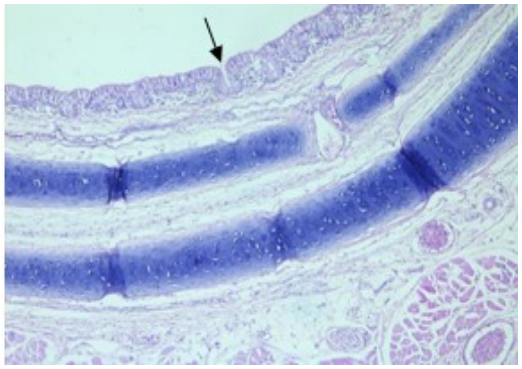

Fig. (7): Trachea of group 3 Treated with doxycyclin after infection of MG) showing activation of goblet cells with pocket formation. H&E X 400

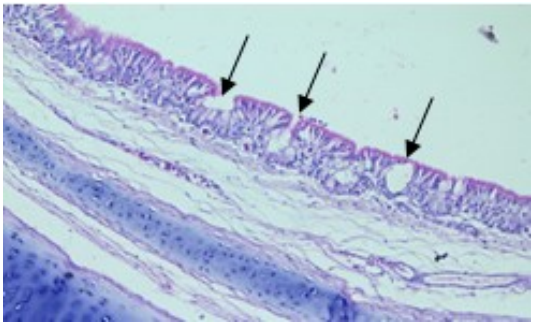

Fig. (8): Trachea of group 4 (reated with tiamulin before infection of MG) showing pronounced activation of goblet cells. H&E20

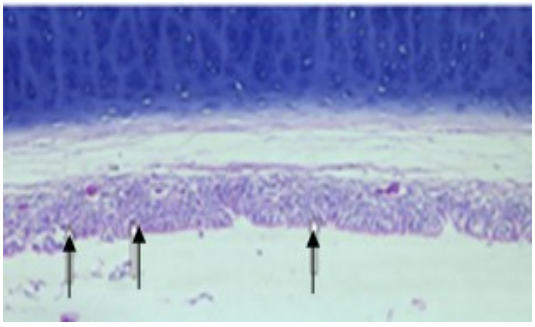

Fig. (9): Trachea of group 5 showing marked epithelial hyperplasia. H&E X 200

Table (8). Results of histopathology of different groups of experiment

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-	-	++	-	-	++
	Sub mucosa and lamina propria Congestion of blood vessels	-	+++	-	+	++
	-Proliferation of inflammatory cell	-	+++	+	-	-
	Thickening	-	++	+	-	++
	Muscular layer Edema of muscular layer	-	++	+	-	++
Bronc-hiole	Epithelial hyper plasia	-	+++	-	-	-
	Proliferation of inflammatory cell	-	+++	-	-	++

Gr.: group
+++: high positive
++: moderate positive
+: weak positive
-: Negative

Discussion

M. gallisepticum colonizes its host mainly via the mucosal surfaces of the respiratory tract, causing air sacculitis within a few days (**Much *et al.,* 2002**), and disseminates throughout the body. This systemic infection is reflected by the high rate of *M. gallisepticum* re-isolation from inner organs such as the liver, heart, spleen, or kidney (**Much *et al.,* 2002**) and by its detection inside and at the surface of red blood cells of experimentally infected birds **(Vogel *et al*., 2008).**

The present study started with collection of samples from three commercial layer flocks in Menofia Governorate were examined for the detection of mycoplasma infection, two flocks suffered from respiratory signs and the recovery rates were 16% and 20% respectively. The incidence of infection was 8% in the apparently healthy flock. 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 Baladi breed (45w) (18.33%) in Kaluobia Governorate.

Broiler flocks suffered from respiratory manifestation were positive for mycoplasma isolation with percentage of 33.6%, while the apparently healthy flocks were positive with a percentage of 12.5%. **Sokkar *et al.,* (1986)** take swabs from trachea, sinuses and air sacs from 50 chickens of different ages and revealed the presence of *M. gallisepticum* in 12 chickens (24%).

In balady breeds 27 out 115 samples were positive for Mycoplasma with percentage of 23.47 % , also these results were agreed with **Abd El -Gwad (2005)** while the apparently healthy flock was negative.

In breeders, one flock was examined for *MG* and *MS*. This flock was suffering from respiratory manifestation, swelling in joints and lameness. The results for *MG* was 20% and 4% for *MS*. The results were in agreement with **El-Shater (1986)** who isolated *MG* (16.7%) of the examined birds and disagreed with **Ulgen and Kahraman (1993)** who isolated MG from layer and breeder with a percentage of 34.9%.

PCR is rapid, sensitive and accurate test which depend on the detection of DNA even the non-viable mycoplasma. In the present work, PCR assay *16s*rRNA gene was used for screening of the isolates and then confirmed with detection of mgc2 virulent gene for *Mycoplasma gallisepticum*. Also, *16srRNA* gene and virulent *vlhA* gene could be detected in MS isolates. The obtained results were agreed with Garcia et al., 2005 who compared and evaluate various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. They found that cydtadesin membrane surface protein (*mgc*2) 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 *Mycoplasma synoviae*, they detected a specific PCR fragment at 1100 bp. In the current study, one *Mycoplasm gallisepticum* field isolate (*mgc*2 gene) was sequenced, analyzed and compared with data base on GenBank. The results were in similar with Rab1-08 strain 100% identity.

A laboratory experiment was done to study the pathogenicity of *Mycoplasma gallisepticum* field strain (Nouh-C15-*mgc*2) 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 effective when given as prophylactic before infection, while doxycycline was effective for treatment of Mycoplasma infection.

A decrease in body weight was noticed in all infected groups compared with negative control group during the whole period of the experiment, **Stipkovits (1979)** mentioned that, meat and egg production of infected birds were decreased. There was no mortality noticed in chickens inoculated with MG. These results were in agreement with **Mohamed (1997)** who mentioned that there was no mortalities in chickens experimentally infected with MG.

Eissa *et al.,* 2009 concluded that treatment of chickens infected with MG by Tiamulin resulted in increase of body weight and decrease of pathological lesions of internal organs; also

they mentioned that five birds out of ten and PCR were positive at the second and third week post treatment.

Conclusion

Identification of *Mycoplasma gallisepticum* (MG) and *Mycoplasma Synoviae* (MS) using PCR is more accurate than traditional identification.

Incidence of infection by MG in broiler was higher than layer, Saso, balady and breeder due to highly resistant of these breeds to infection. Using of Tiamulin and doxycycline gave good result in controlling of mycoplasma.

References

Abd El Gawad Rania, H. (2005). Detection of mycoplasma infection in native breed layers by recent techniques. M.V.Sc. Thesis, Kafr El Shiekh, Fac. Vet. Med.., Tanta University.

Bancreoft, J.D. and Gamble, M. (2008). Bacteriology culture media (chapter 12) in Whitman K, A, and MacNair N.G, (eds.): finfish and shelfish, Bacteriology Manual: Techniques and procedures. Lowa State press, Ames, Lowa: 89-90.

Buim, M.; Mettifogo, E.; Timenetsky, J.; Kleven, S. and Ferreira, A. (2009). Epidemiological survey on *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by multiplex PCR in commercial poultry pesq. Vet. Bras. 27: 552-556.

Eissa, S.I.; Dardeer, M.A. Hassan, A.M.; Abd Elaziz, E.E. and Al-zeftawy, N.M. (2009). In vitro and in Vivo Evaluation of some antimicrobials activity against *Mycoplasma gallisepticum* isolates from day old chicks in Egypt. Zag. Vet. J. 37(5): 82-91.

El-Shatter, S.A.A. (1986). Some studies on chronic respiratory disease in fowls Ph.D. Thesis, Poultry department, Faculty of Vet. Med., Assiut - Univ.

Erno, H. and Stipkovits, L. (1973). Bovine Mycoplasmas, cultural and biochemical studies. Acta-Vet. Scand., 14: 450-463.

Evans, J.; Thornton, D. and Branton, S. (2009). Diagnosis of *Mycoplasma gallisepticum* from a Broiler Breeder Flock: Comparison of Three Diagnostics J.D Methods. International Journal of Poultry Science, 8: 104-107.

Fabricant, J. and Freundt, E.A. (1967). Importance of extension and standardization of mycoplasmas. Ann. N.Y. Acad. Sci. 143P: 50-58

Feberwee (2005). Comparison of culture, PCR and different serologic tests for detection *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection. Avian Dis, 49, 260-268 .

Freundt, E.A.; Anderson, B.E.; Erno, N.; Kunze, M. and Blank, F.T. (1973). The sensitivity of Mycoplasmatales to sodium polyanethol sulphate and digitonin. Zbl Back. Parasit. Infehtkr. Hyg. L. Abt. Drig. A. 225: 104-112.

Frey, M.C.; Hanson, R.P. and Anderson, D.P. (1968). A medium for the isolation of avian Mycoplasma. Ann. Vet. Res., 29: 2164 -2171.

Garcia, M.; Ikutan, N.; Leveisohn, S. and Kleven, S.H. (2005). Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. Avian Dis, Mar; 49 (11): 125-132.

Hannan, P.C.T. (2000). Guidelines and recommendation for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary mycoplasma species. Vet. Res., 31: 373-395.

Hammond, P.P.; Ramerez, A.S.; Morrow, C.J. and Bradbury, J.M. (2009). Development, evaluation of an improved diagnostic PCR for Mycoplasma synoviae using primer located in the haemagglutinin encoding gene vlh A and its value for strain typing. Vet. Micrbiol, El-Sevier, 2009 -6-(1-7).

Kleven, S.H. (1997). *Mycoplasma synoviae* infection,. In B.W. Calnek, H.J. Barnes, C.

W. Beard, L.R. McDougal, and Y.M. Saif (ed.), Diseases of poultry, 10th ed. Iowa State University Press, Ames., p. 220–228.

Mohamed, Z.R. (1997). Some studies on *Mycoplasma gallisepticum* in chicken in Egypt. M.V. Sci. Thesis, Poultry diseases Department, Faculty of Vet. Med., Zagazig University.

Much, P.; Winner, F.; Stipkovits, L.; Rosengarten, R. and Citti, C. (2002). *Mycoplasma gallisepticum*: influence of cell invasiveness on the outcome of experimental infection in chickens. FEMS Immunol. Med. Microbiol. 34: 181–186**.**

OIE (2008). Manual of standards for diagnostic tests and vaccine ,World Organization for Animal Health (OIE) 6thEd., Paris, France.

Sabry, M.Z. and Ahmed, A.A. (1975). Evaluation of media and cultural procedurefor the primary isolation of Mycoplasma from female genitalia farmanimals. J. Egypt. Vet. Med. Assoc., 35: 18-36.

Sokkar, I.M.; Soliman, A.M. Mousa, S. and ElDemerdash, M.Z.. (1986). Invitro sensitivity of mycoplasmas associated bacteria isolated from chickens, turkeys, and ducks at the area of Upper Egypt. Vet Bull., 56(8) p. 683.

Sprygin, A.; Andeychuk, D.; Elatkin, N.; Zinyakov, N.; Kolosov, S.; Mudrak, N.; Irza, V.; Drygin, V.; Borisov, A. and Perevozchikova, N. (2010). Genetic diversity of *Mycoplasma gallisepticum* field isolates using partial sequencing the PvPA gene fragment. Avian Dis. 54 (2): 899-904.

Stipkovits, L. (1979). The pathogenicity of avian mycoplasmas. Zentralbl Bakteriol Orig A245 (1-2): 171-83.

Ulgen, M. and Kahraman, M. (1993). Comparative bacteriological serological studies on avian Chronic Respiratory Disease (CRD) Veterinarium,4(2): 33-35.

Vogl, G.; Plaickner, A.; Szathmary, S.; Stipkovits, L.; Rosengarten, R. and Szostak, M.P. (2008). *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. Infect. Immun. 76: 71–77.

Yilmaz, F.; Timurkaan, N.; Kilic, A.; Kalender, H. and Kilinc, U. (2011). Detection of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* in chickens by immunohistochemical PCR and culture methods. Rev. Med. Vet. 162: 79-86 .

Zhao, S. and Yamamoto, R. (1993). Detection of *Mycoplasma synoviae* by polymerase chain reaction. Avian Pathology 22: 533-542.