OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2018.

Research Article
Molecular Identification and Sequencing of *Mycoplasma gallisepticum* Recovered from Broilers in Egypt

[1]Gamal A.M. Younis, [2]Rania H. AbdElgawad, [1]Rasha M. Elkenany and [3]Amira F. Glal

[1]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
[2]Department of Mycoplasma Research, Animal Health Research Institute, Dokki, Giza, Egypt
[3]Veterinarian, Mansoura, Egypt

Abstract

Background and Objectives: Avian mycoplasmosis, particularly *Mycoplasma gallisepticum* (MG) is one of the infectious diseases associated with economic losses in Egyptian poultry industry. Thus, this study was aimed to determine the prevalence, serological identification, molecular characterization, sequencing and minimum inhibitory concentration of *M. gallisepticum* isolated from diseased broilers in Egypt. **Materials and Methods:** A total of 351 samples (227 tissue samples "tracheas and air sacs" and 124 tracheal swabs) and 71 sera were collected from diseased broilers. The conventional (isolation and biochemical) and molecular methods (PCR) were performed for detection of *M. gallisepticum* and virulence-associated gene (*mgc2*). The serum plate agglutination (SPA) test and enzyme-linked immunosorbent assay (ELISA) were applied on sera for determination of the presence of antibodies against *M. gallisepticum*. The minimal inhibitory concentration test (MIC) was used to determine the sensitivity of two sequenced *M. gallisepticum* strains to anti-mycoplasma agents. **Results:** The total recovery rate of Mycoplasma from 351 samples from broilers was 45.29% (159) in which *M. gallisepticum* showed a prevalence of 62.89% (100/159). Serological identification of *M. gallisepticum* in 71 collected sera using SPA and ELISA were 54.9 and 40.8% with the highest geometric mean titer of ELISA for *M. gallisepticum* (699.08 and 495.92). Molecular characterization of Mycoplasma using PCR showed that 50% (3/6) of tested isolates were identified as *M. gallisepticum* based on *16SrRNA*. Also, the *mgc2* gene was detected in 50% (3/6) *M. gallisepticum* isolates. Two positive PCR *mgc2* specific genes of *M. gallisepticum* isolates were subjected to gene target sequencing (GTS) to verify that these two isolates were *M. gallisepticum*. The minimal inhibitory concentration test (MIC) was applied to determine the sensitivity of these two sequenced *M. gallisepticum* strains to anti-mycoplasma agents. The first *M. gallisepticum* isolate was sensitive to tilmicosin, tiamulin and spiramycin. The second *M. gallisepticum* isolate showed sensitivity to tiamulin, spiramycin and tilmicosin. **Conclusion:** These results summarized the necessity of monitoring the Egyptian poultry farms for avian mycoplasmosis. Also, further studies are required for controlling of mycoplasma in all stages of the poultry industry production chain to avoid different losses in Egypt.

Key words: *M. gallisepticum*, ELISA, PCR, *mgc2* gene, minimal inhibitory concentration, Egyptian poultry farms, avian mycoplasmosis, diseased broiler

Citation: Gamal A.M. Younis, Rania H. AbdElgawad, Rasha M. Elkenany and Amira F. Glal, 2018. Molecular identification and sequencing of *Mycoplasma gallisepticum* recovered from broilers in Egypt. Pak. J. Biol. Sci., CC: CC-CC.

Corresponding Author: Rasha M. Elkenany, Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, P.O. Box 35516, Mansoura, Egypt Tel: +2-01090226696

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mycoplasma is a very small prokaryote, tends to be quite host-specific. It is found in human, many animal species and insects. Avian mycoplasmosis caused by *Mycoplasma* has designated as a notifiable disease by the World Organization for Animal Health (OIE) because of its economic losses in broilers. These losses resulted from the condemnation of carcasses, reduction in weight gain, increased mortality and losses in breeders and layers[1,2].

Mycoplasma gallisepticum (*MG*) is frequently present as one of the major agents in multifactorial disease complex. The *M. gallisepticum* is associated with exclusion or reduction of carcasses, enhancing in feed conversion ratio and economical losses of vaccination or drug prevention. The *M. gallisepticum* is also commonly known as airsacculitis and often infra-orbital sinusitis causes in turkeys and chronic respiratory disease (CRD) in chickens with a wide variety of clinical signs[3].It predisposes the birds to the action of *Escherichia coli*, vaccinal strains of Newcastle disease or Infectious Bronchitis, which lead to chronic respiratory disease (CRD)[3].

Three main approaches (isolation, serological and molecular identification) were used for the diagnosis of avian *Mycolplasma*. The isolation method was complicated, costly and time-consuming[4]. The serological identification was useful for early and rapid diagnosis such as Serum Plate Agglutination (SPA) assay which was used as a screening test and Enzyme-Linked Immunosorbent Assay (ELISA) which is considered as the preferred method for antibodies detection[5]. Serological screening is still in widespread use, but may not detect the subclinical infection. Now-a-days, a molecular technique through detection of organism nucleic acid using Polymerase Chain Reaction (PCR) is widely used and improves the diagnosis of *Mycoplasma* in chickens[6].

Furthermore, different *M. gallisepticum* strains exhibit wide variations in virulence. Differences in virulence between *M. gallisepticum* isolates belonging to the same strain may also be seen with variations in culture methods, passage levels and inoculation routes and dosages (289). The cytadhesin membrane surface protein of *M. gallisepticum* is one of the important virulence factor encoded by *mgc2* gene, which recently demonstrated to be necessary for attachment of *Mycoplasma* cells and their gliding motility. The last researches were applied to confirm their characterization of *M. gallisepticum* isolates through sequencing of *mgc2* gene to be sure that their *M. gallisepticum* isolates were field or vaccinal strains and to make an evaluation to vaccine programs[7-9].

The antimicrobial chemotherapy has an important role in the treatment of *M. gallisepticum* infections. In contrast, *Mycoplasma* has been considered as a type of delicate and slowly growing bacteria. *Mycoplasma* needs special growth media and culturing condition, so study on *Mycoplasma* is difficult in many instances. Consequently, minimal inhibitory concentration test (MIC) is widely used for determining the most effective antibiotics against *Mycoplasma* in poultry farms to eliminate *Mycoplasma* infection[10-12].

Epidemiological studies of *M. gallisepticum* in avian populations has concentrated mostly on the poultry farms and can assistance also in the prevention and controlling of *M. gallisepticum* infection. Therefore, the purpose of this study was to determine the prevalence, serological identification, molecular characterization, sequencing and minimum inhibitory concentration of *M. gallisepticum* isolated from diseased broilers in Egypt.

MATERIALS AND METHODS

Ethical approval: This investigation was conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the authority of the Faculty of Veterinary Medicine, Mansoura University, Egypt. All institutional and National guidelines for the care and use of animals were followed according to the Egyptian Medical Research Ethics Committee (No.14-26).

Sampling: A total of 351 samples (227 tissue samples "tracheas and air sacs" and 124 tracheal swabs) and 71 sera were collected from diseased broilers suffering from respiratory manifestations with age 1-30 days from six different farms located at different districts at Dakahlia govern orate in Egypt from November 2016 to April 2017, under complete aseptic conditions.

Isolation and identification of *Mycoplasma*: The isolation of *Mycoplasma* from broiler tissue samples and tracheal swabs was carried out as previously described[13]. Genus determination and biochemical characterization of *Mycoplasma* was performed according to Erno and Stipkovits[14].

Serological identification of *M. gallisepticum*: The collected serum samples were subjected to serum plate agglutination (SPA) assay and Enzyme labeled immunosorbent assay (ELISA) test for detection of specific *M. gallisepticum* antibodies. The SPA test was conducted as stated by OIE[15]. In this test, an equal amount of *M. gallisepticum* antigen (Lilli test *M. gallisepticum* RSA Antigen, Manufactured by Lillidale Diagnostics, Pig Oak Farm, Holt, Wimborne, Dorset) and broiler serum were placed and

Table 1: Primer sequences and cycling conditions used for detection of *M. gallisepticum*

Primer	Sequence	Cycling	References
16SrRNA	MG-14F:5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3' MG-13R:5'GCT-TCC-TTG-CGG-TTA-GCA-AC-3'	Initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation, annealing, extension at 94°C for 30 sec; 55°c for 30 sec and 72°C for 60 sec, respectively, then final extension at 72°C for 5 min	OIE[15]
mgc2	F:5'- CGC AAT TTG GTC CTA ATC CCC AAC A- 3' R: 5'- TTA ACC CAC CTC CAG CTT TAT TTC C-3	Initial denaturation at 95°c for 3 min followed by 35 cycles of denaturation, annealing, extension at 94°c for 30 sec; 58°c for 40 second 72°c for 60 sec, respectively, then final extension at 72c for 10 min	Garcia *et al.*[16]

thoroughly mixed on a glass plate. The formation of clumps within 2 min is the characters of positive reaction in SPA test.

Moreover, a commercial *Mycoplasma* ELISA kit (Synbiotics corporation, San Diag, CA 92127, USA) was used to detect specific antibodies against *M. gallisepticum* in collected broiler serum samples. The ELISA test procedures were carried out according to the manufacturer's recommendations with help of full automatic plate washer Model ELX800 and ELISA Reader (Bio-Tek, ELX-800-650).

Molecular identification of *M. gallisepticum*: The polymerase chain reaction (PCR) assay targeting *16SrRNA* gene (a determinant of *M. gallisepticum* species) and *mgc2* gene (virulence-associated gene) was applied. The genomic DNA was extracted using Thermo-genomic DNA extraction kit (Lithuania) following the manufacturer's recommendations. The extracted DNA samples were subjected to PCR amplification using specific primers and specific profiles as shown in Table 1[15,16]. The PCR amplified products were electrophoresed through 1.5% agarose gel, visualized by UV fluorescence and then photographed.

Determination of *mgc2* gene sequence in *M. gallisepticum*: The purification of amplified products was done from two representative strains using a Gene JET PCR purification kit (Fermentas, EU) and sequenced by Macro gene Company, South Korea. The sequences of these strains were compared with other strains on GenBank by using BLAST 2.0 and PSI-BLAST search programs, National Center for Biotechnology Information (NCBI). The obtained nucleotide sequences comparisons and their multiple alignments with reference strains as well as the deduction of amino acid sequences were done using the BioEdit sequence alignment editor. The Clustal X software for multiple sequence alignment, clustal W software for multiple sequence alignment[17]. Clustal V and MegAlign (DNASTAR, Lasergene, Version 7.1.0, USA) were used[18]. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on Clustal W. Bootstrapping values were calculated using a random seeding value of 111[17]. Clustal V was used when end gaps were faced. Sequence divergence and identity percentage were calculated by MegAlign. The structural character of our protein sequence was identified by Protean (DNASTAR, Lasergene, Version 7.1.0. USA)[19].

Estimation of minimal inhibitory concentration (MIC): The degree of two representative strains of *M. gallisepticum* sensitivity to most widely used anti-mycoplasma drugs in poultry farms was evaluated by a Minimal inhibitory concentration (MIC) assay method, as previously described[20]. The following anti-mycoplasma drugs were tested; Tylosin and Tilmicosin 25% (ATCO), Tiamulin 45% (Delta vet trading), Lincomycin (Lincol), Doxycycline (Royal dox 50%) and Spiramycin (Royl link Int).

RESULTS

Prevalence of *Mycoplasma* in broilers: The recovery rate of *Mycoplasma* isolated from broilers suffering from respiratory signs with age 1-30 day from six different farms at Dakahlia governorate, Egypt was 45.29% (159/351), by which *M. gallisepticum* showed a prevalence of 62.89% (100/159) (Table 2). Farms numbers (1 and 2) showed the highest occurrence of *Mycoplasma* (51.42 and 53.48%, respectively) with positive arginine deamination, film and spot formation and absence of glucose fermentation. On the other hand, farms numbers (3, 4 and 6) displayed *Mycoplasma* prevalence (51.31, 47.05 and 43.93%, respectively) with positive glucose fermentation test, negative arginine deamination and negative film and spot tests, which indicated *M. gallisepticum* isolates in such farms. The samples collected from farm number (5) was negative for Mycoplasma isolates.

Serological identification of *M. gallisepticum*: The SPA and ELISA tests were applied for serological identification of serum samples. Positive *M. gallisepticum* antibodies for SPA and ELISA tests were (54.9%) and (40.8%), respectively. Geometric mean titer (GMT) was calculated for *M. gallisepticum* positive serum samples subjected to ELISA test. In particular, farms numbers (3 and 4) revealed the highest GMT of ELISA for *M. gallisepticum* (699.08 and 495.927, respectively), while farms numbers (2 and 5) presented the lowest GMT of ELISA for *M. gallisepticum* (85.724 and 11.423, respectively) (Table 3).

Table 2: Prevalence and biochemical characterization of *Mycoplasma* recovered from broilers in six different farms

Farm No.	Breed	Age (day)	Housing system	Number of samples			Number of positive Mycoplasma isolates		Prevalence (%)	Biochemical characterization			Number of positive MG isolates	
				Tracheal swabs	Tissues		Tracheal swabs	Tissues		Glucose fermentation	Arginine deamination	Film and spot formation	Tracheal swabs	Tissues
1	Copp	23	Semiclosed	42	28		21	15	51.42	-	+	+	0	0
2	Indin river	1	Semiclosed	-	43		-	23	53.48	-	+	+	0	0
3	Arbor	28	Semiclosed	49	27		26	13	51.31	+	-	-	26	13
4	Copp	18	Closed	33	35		22	10	47.05	+	-	-	22	10
5	Ross	3	Semiclosed	0	28		0	0	0.00	-	-	-	0	0
6	Arbor	1	Closed	0	66		0	29	43.93	+	-	-	0	29
Total				124	227		69	90	45.29				48	52
							159/351						100/159 (62.89%)	

MG: *Mycoplasma gallisepticum*

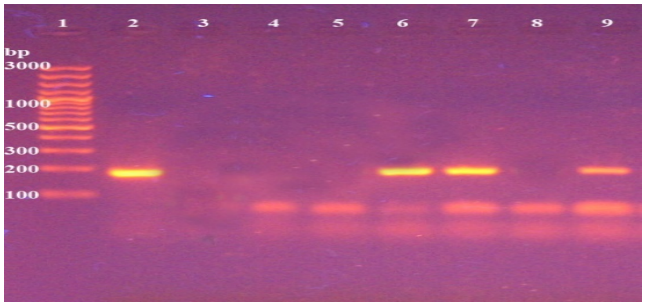

Fig. 1: Electrophoresis gel of *16SrRNA* gene of *M. gallisepticum* isolated from broilers at 185 bp. Lane 1: DNA Ladder, Lane 2: Control positive, Lane 3: Control negative, Lanes 4, 5, 8: Negative samples, Lanes 6, 7, 9: Positive samples

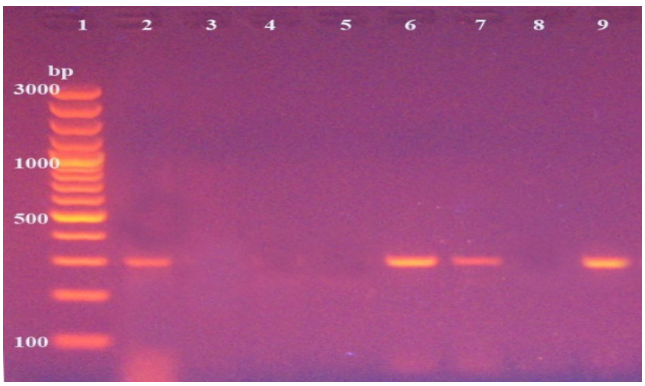

Fig. 2: Electrophoresis gel of *mgc2* gene of *M. gallisepticum* isolated from broilers at 300 bp. Lane 1: DNA Ladder, Lane 2: Control positive, Lane 3: Control negative, Lanes 4, 5, 8: Negative samples, Lanes 6, 7, 9: Positive samples

Molecular identification of *M. gallisepticum*: The PCR assay as confirmatory test identified three *M. gallisepticum* isolates (3/6, 50%) in three different farms depending on *16SrRNA* gene (Fig. 1). Moreover, three (50%) of six *M. gallisepticum* isolates displayed the presence of the *mgc2* gene (Fig. 2).

Sequence analysis of *mgc2* gene in *M. gallisepticum* strains: Sequencing of *mgc2* gene from two purified PCR products of two *M. gallisepticum* strains isolated from broilers in two different farms was performed. These two sequences were submitted to GenBank database under the accession number [MF 77 38 76 and MF 77 38 77] and designation (RAG-1-MG-CK-Eg017 and RAG-2-MG-CK-EG 017), respectively. Moreover, nucleotide phylogenetic tree of *mgc2* specific gene of *M. gallisepticum* isolates (RAG-1-MG-CK-Eg017 and RAG-2-MG-CK-EG 017) recovered from Farm numbers 3 and 4

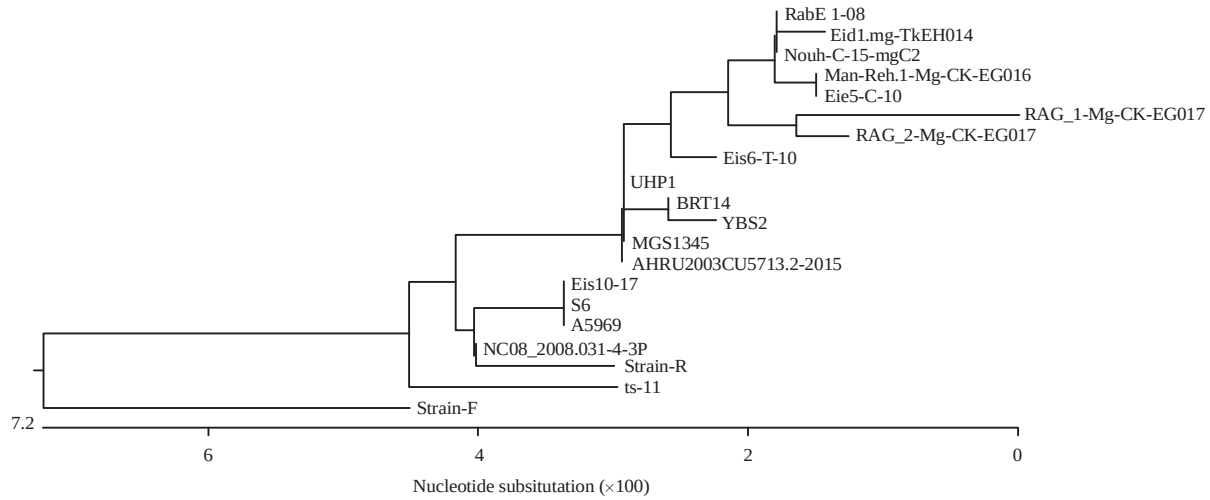

Fig. 3: Phylogenetic tree of nucleotides of *mgc2* gene of *M. gallisepticum* (RAG-1-MG-CK-EG017 & RAG-2-MG-CK-EG 017) compared with reference strains

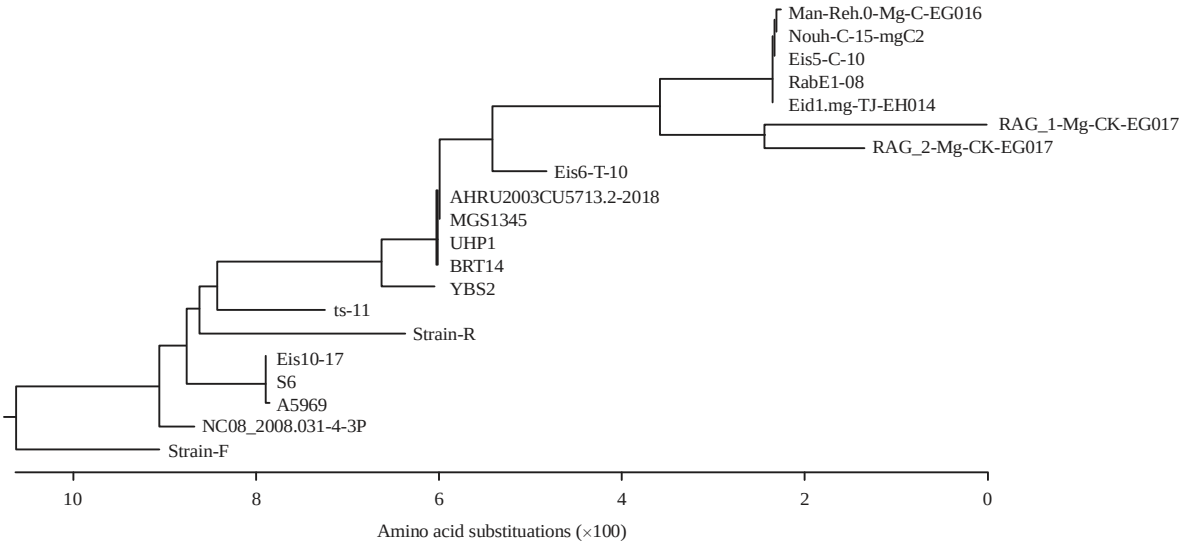

Fig. 4: Phylogenetic tree of amino acids of mgc2 gene of *M. gallisepticum* (RAG-1-MG-CK-EG017 &RAG-2-MG-CK-EG017) compared with reference strains

Table 3: Geometric mean titer (GMT) of ELISA and Serum plate agglutination (SPA) for *Mycoplasma gallisepticum* in collected sera (n = 71) from six different farms

		SPA		ELISA			
Farm No.	No of samples	Negative	Positive	Negative	Suspect	Positive	GMT of ELISA
1	7	3	4	-	4	3	394.299
2	13	6	7	8	-	5	85.724
3	14	4	10	1	5	8	699.08
4	17	8	9	3	8	6	495.927
5	10	7	3	7	1	2	11.423
6	10	4	6	2	3	5	367.544
Total	71	32(45.1%)	39 (54.9%)	21(29.8%)	21(29.8%)	29(40.8%)	

showed similarity ranged 96-100% with reference and field strains (Table 4) (Fig. 3), while the amino acids phylogenetic tree of the same gene of these isolates showed similarity ranged from 98-100% with reference and field strains (Table 5, Fig. 4). The sequence and phylogenetic analysis proved that these two isolates were *M. gallisepticum*.

Table 4: Identity percent of nucleotides sequencing of MG (RAG-1-MG-CK-EG017 and RAG-2-MG-CK-EG 017) *mgc2* gene compared with reference and field strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
1	■	95.4	95.3	94.3	94.3	94.3	94.3	94.3	93.1	93.1	93.1	93.1	92.0	88.5	88.5	89.7	88.5	87.4	89.7	86.2	1	RAG_1-MG-CK-EG017
2	4.8	■	96.5	96.6	96.6	96.6	96.6	96.6	96.4	95.4	95.4	95.4	94.3	90.8	90.8	92.0	90.8	90.8	92.0	88.5	2	RAG_2-MG-CK-EG017
3	4.8	3.6	■	100.0	100.0	95.3	100.0	100.0	96.5	96.5	96.5	96.5	95.3	91.9	91.9	93.0	91.9	90.7	93.0	89.5	3	Man-Reh.1-MG-CK-EG016
4	6.0	3.5	0.0	■	100.0	95.4	100.0	100.0	96.6	96.6	96.6	96.6	95.4	92.0	92.0	93.1	92.0	90.8	93.1	89.7	4	Nouh-C-15-mgC2
5	6.0	3.5	0.0	0.0	■	95.4	100.0	100.0	96.6	96.6	96.6	96.6	95.4	92.0	92.0	93.1	92.0	90.8	93.1	89.7	5	Eis5-C-10
6	6.0	3.5	4.8	4.8	4.8	■	95.4	95.4	98.9	98.9	98.9	98.9	97.7	94.3	94.3	95.4	94.3	93.1	95.4	92.0	6	Eis6-T-10
7	6.0	3.5	0.0	0.0	0.0	4.8	■	100.0	96.6	96.6	96.6	96.6	95.4	92.0	92.0	93.1	92.0	90.8	93.1	89.7	7	RabE1-08
8	6.0	3.5	0.0	0.0	0.0	4.8	0.0	■	96.6	96.6	96.6	96.6	95.4	92.0	92.0	93.1	92.0	90.8	93.1	89.7	8	Eid1.mg-TK-EG014
9	7.2	4.8	3.6	3.5	3.5	1.2	3.5	3.5	■	100.0	100.0	100.0	98.9	95.4	95.4	96.6	95.4	94.3	96.6	93.1	9	AHRU2003CU5713.2-2015
10	7.2	4.8	3.6	3.5	3.5	1.2	3.5	3.5	0.0	■	100.0	100.0	98.9	95.4	95.4	95.6	95.4	94.3	96.6	93.1	10	MG51345
11	7.2	4.8	3.6	3.5	3.5	1.2	3.5	3.5	0.0	0.0	■	100.0	98.9	95.4	95.4	96.6	95.4	94.3	96.6	93.1	11	UHP1
12	7.2	4.8	3.6	3.5	3.5	1.2	3.5	3.6	0.0	0.0	0.0	■	98.9	95.4	95.4	96.6	95.4	94.3	96.6	93.1	12	BRT14
13	8.5	6.0	4.8	4.8	4.8	2.3	4.8	4.8	1.2	1.2	1.2	1.2	■	95.4	95.4	96.6	95.4	94.3	96.6	93.1	13	YBS2
14	12.5	9.8	8.6	8.5	8.5	6.0	8.5	8.5	4.8	4.8	4.8	4.8	4.8	■	100.0	98.9	100.0	96.6	96.6	95.4	14	Eis10-17
15	12.5	9.8	8.6	8.5	8.5	6.0	8.5	8.5	4.8	4.8	4.8	4.8	4.8	0.0	■	98.9	100.0	96.6	96.6	95.4	15	S6
16	11.2	8.5	7.3	7.2	7.2	4.8	7.2	7.2	3.5	3.5	3.5	3.5	3.5	1.2	1.2	■	98.9	97.7	97.7	96.6	16	NC08_2008.031-4-3P
17	12.5	9.8	8.6	8.5	8.5	6.0	8.5	8.5	4.6	4.8	4.8	4.8	4.8	0.0	0.0	1.2	■	96.6	96.6	95.4	17	A5969
18	13.9	9.8	10.0	9.8	9.8	7.2	9.8	9.8	6.0	6.0	6.0	6.0	6.0	3.5	3.5	3.5	3.5	■	95.4	94.3	18	Strain-R

Table 5: Identity percent of amino acids sequencing of *MG*(RAG-1-MG-CK-EG017 and RAG-2-MG-CK-EG 017) *mgc2* gene compared with reference and field strains

Percent identity

	1	2	3	4	5	6	T	8	9	10	11	12	13	14	15	16	17	18	19	20		
1	■	98.0	97.7	97.3	97.7	97.7	97.3	97.0	97.0	97.0	97.0	96.7	96.3	95.0	95.0	95.7	95.0	94.3	94.3	90.3	1	RAG_1-Mg-CK-EG017
2	2.0	■	98.3	98.7	98.3	99.0	98.7	98.3	98.3	98.3	98.3	98.0	97.7	96.3	96.3	97.0	96.3	96.0	95.3	91.7	2	RAG_2-Mg-CK-EG017
3	2.4	1.7	■	99.7	100.0	98.0	99.7	99.3	98.7	98.7	98.7	98.3	98.0	96.7	96.7	97.3	96.7	96.3	95.7	92.0	3	Man-Reh.1-Mg-CK-EG016
4	2.7	1.3	0.3	■	99.7	98.3	100.0	99.7	99.0	99.0	99.0	98.7	98.3	97.0	97.0	97.7	97.0	96.7	96.0	92.3	4	Nouh-C-15-mgC2
5	2.4	1.7	0.0	0.3	■	98.0	99.7	99.3	98.7	98.7	98.7	98.3	98.0	96.7	96.7	97.3	96.7	96.3	95.7	92.0	5	Eis5-C-10
6	2.4	1.0	2.0	1.7	2.0	■	98.3	98.0	99.3	99.3	99.3	99.0	98.7	97.3	97.3	98.0	97.3	97.0	96.3	92.7	6	Eis6-T-10
7	2.7	1.3	0.3	0.0	0.3	1.7	■	99.7	99.0	99.0	99.0	98.7	98.3	97.0	97.0	97.7	97.0	96.7	96.0	92.3	7	RabE1-08
8	3.1	1.7	0.7	0.3	0.7	2.0	0.3	■	98.7	98.7	98.7	98.3	98.0	96.7	96.7	97.3	96.7	96.3	95.7	92.0	8	Eid1.mg-TK-EG014
9	3.1	1.7	1.3	1.0	1.3	0.7	1.0	1.3	■	100.0	100.0	99.7	99.3	98.0	98.0	98.7	98.0	97.7	97.0	93.3	9	AHRU2003CU5713.2-2015
10	3.1	1.7	1.3	1.0	1.3	0.7	1.0	1.3	0.0	■	100.0	99.7	99.3	98.0	98.0	98.7	98.0	97.7	97.0	93.3	10	MGS1345
11	3.1	1.7	1.3	1.0	1.3	0.7	1.0	1.3	0.0	0.0	■	99.7	99.3	98.0	98.0	98.7	98.0	97.7	97.0	93.3	11	UHP1
12	3.4	2.0	1.7	1.3	1.7	1.0	1.3	1.7	0.3	0.3	0.3	■	99.7	97.7	97.7	98.3	97.7	97.3	96.7	93.0	12	BRT14
13	3.8	2.4	2.0	1.7	2.0	1.3	1.7	2.0	0.7	0.7	0.7	0.3	■	97.3	97.3	98.0	97.3	97.0	96.3	92.7	13	YBS2
14	5.2	3.8	3.4	3.1	3.4	2.7	3.1	3.4	2.0	2.0	2.0	2.4	2.7	■	100.0	99.3	100.0	98.3	97.0	94.3	14	Eis10-17
15	5.2	3.8	3.4	3.1	3.4	2.7	3.1	3.4	2.0	2.0	2.0	2.4	2.7	0.0	■	99.3	100.0	98.3	97.0	94.3	15	S6
16	4.5	3.1	2.7	2.4	2.7	2.0	2.4	2.7	1.3	1.3	1.3	1.7	2.0	0.7	0.7	■	99.3	99.0	97.7	94.3	16	NC08_2008.031-4-31.
17	5.2	3.8	3.4	3.1	3.4	2.7	3.1	3.4	2.0	2.0	2.0	2.4	2.7	0.0	0.0	0.7	■	98.3	97.0	94.3	17	A5969
18	5.5	4.1	3.8	3.4	3.8	3.1	3.4	3.8	2.4	2.4	2.4	2.7	3.1	1.7	1.7	1.0	1.7	■	96.7	93.3	18	Strain-R
19	5.9	4.8	4.5	4.1	4.5	3.8	4.1	4.5	3.1	3.1	3.1	3.4	3.8	3.1	3.1	2.4	3.1	3.4	■	93.3	19	ts-11
20	10.4	8.9	8.5	8.1	8.5	7.8	8.1	8.5	7.0	7.0	7.0	7.4	7.7	5.9	5.9	5.9	5.9	7.0	7.0	■	20	Strain-F
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		

Table 6: MIC of some antibiotics against *M. gallisepticum* (RAG-1-MG-CK-EG017) strain

	MIC (mg mL^{-1})	
Antibiotic drugs	First reading	Second reading
Tilmicosin (25%)	0.06	0.25
Tiamulin (45%)	0.12	0.25
Tylosin (25%)	0.50	1.00
Spiramycin (150000 IU g^{-1})	0.25	0.50
Doxycycline (50%)	1.00	2.00
Lincomycin (40%)	0.50	1.00

Table 7: MIC of some antibiotics against *M. gallisepticum* (RAG-2-MG-CK-EG017) strain

	MIC (mg mL^{-1})	
Antibiotic drugs	First reading	Second reading
Tilmicosin (25%)	0.25	1.00
Tiamulin (45%)	0.12	0.25
Tylosin (25%)	0.50	1.00
Spiramycin (150000 IU g^{-1})	0.12	0.50
Doxycycline (50%)	0.50	1.00
Lincomycin (40%)	1.00	2.00

Minimal inhibitory concentration (MIC): These two sequenced isolates (RAG-1-MG-CK-Eg017 and RAG-2-MG-CK-EG 017) were subjected to MIC test using six antibacterial agents. The first isolate showed sensitivity to Tilmicosin, Tiamulin and Spiramycin, respectively, while another isolate displayed sensitivity to Tiamulin, Spiramycin and Tilmicosin, respectively (Table 6, 7).

DISCUSSION

Avian mycoplasmosis is one of the infectious diseases caused by many species of *Mycoplasma* especially *M. gallise*. The *M. gallisepticum* is commonly involved in chronic respiratory diseases in chicken and infectious sinusitis in turkey[21]. The *M. gallisepticum* infections are often complicated by *Escherichia coli* and/or respiratory viruses as Infectious bronchitis, Avian Paramyxovirus leading to chronic respiratory disease (CRD)[3]. *Mycoplasma* isolation by culture is regarded as the gold standard for diagnosis of avian mycoplasma. Biochemical characterization of *M. gallisepticum* is based on their ability to ferment glucose, but not to hydrolyze arginine and on their lack of phosphatase activity[22]. In the present study, the total recovery rate of Mycoplasma from broiler tissue samples and tracheal swabs collected from six different farms was 45.29% (159/351), in which *M. gallisepticum* prevalence was 62.89% (100/159). This percentage was very close to that mentioned by Dardeer *et al.*[23], who stated that the prevalence of *M. gallisepticum* was 63.49% but higher than that detected by Abd El-Ghany[24], who identified *M. gallisepticum* with the prevalence of 42.66 and 15% in chicken flocks in Egypt, respectively.

Serological tests such as SPA and ELISA tests are useful in monitoring *M. gallisepticum* infection in a flock and for taking prophylactic measures to control *M. gallisepticum* infection early in *M. gallisepticum* free poultry. However, these tests showed a large number of cross-reactions[25]. In this study, the SPA and ELISA tests were applied on 71 collected serum samples. The *M. gallisepticum* positive serological results were 54.9 and 40.8% for SPA and ELISA tests, respectively. These results were lower than that detected by other investigators[21], who deduced that the SPA and ELISA results of antibodies against *M. gallisepticum* in chicken serum were 69.9 and 58.3% in Egypt, respectively. Additionally, a previous study[6]recorded the seroprevalence of *M. gallisepticum* in 56.13 and 64.47% of laying chicken by SPA and ELISA tests in Bangladesh. The obtained SPA results were considered to be higher than those recorded by other researchers[24], who detected positive reactors for *M. gallisepticum* infection in 42.85 and 44% of the Egyptian chickens, respectively.

Confirmation of infection by conventional culture procedures is time-consuming (up to 3 weeks), laborious, expensive, requires personal skills and aseptic condition beside, confusion surrounding the serological tests has been encountered[26]. Therefore, DNA amplification of *M. gallisepticum* using PCR has been performed as a very sensitive, specific and rapid method for detection and identification of *M. gallisepticum*[6]. Species-specific PCR methods targeting the *16SrRNA* genes of *M. gallisepticum* have been developed. The electrophoresis of the PCR products of amplified *16SrRNA* gene of *M. gallisepticum* showed characteristic specific bands at 183 bp to 50% of examined isolates in this study[9,27]. The previous study identified *M. gallisepticum* in 17.8% of chickens in Sudan by PCR[6]. Furthermore, the virulence-associated gene (*mgc2*) was detected in three of six *M. gallisepticum* isolates in this investigation. Similarly, Rasoulinezhad *et al.*[9] detected 48.38% of *M. gallisepticum* in backyards and 16.66% in commercial farms. Eissa *et al.*[28] recognized *mgc2* gene among *M. gallisepticum* strains isolated from chicken and turkey. Also, Moretti *et al.*[29] applied PCR technique for targeting a partial region of the *mgc2* gene to screen various poultry farms in South Africa and Zimbabwe for *M. gallisepticum*.

Recently, PCRs targeting genes which can be used for species identification and strain differentiation (after sequencing of the PCR amplicon) have been developed; these include PCRs targeting the *mgc2* gene of *M. gallisepticum*[30]. The studies were applied as sequence database by targeted genetic sequencing of extracted *Mycoplasma* DNA from commercial poultry types. This sequence data was used in the characterization of *Mycoplasma* and to determine their distribution within the country[27]. Thus, two positive PCR *mgc2*

gene of *M. gallisepticum* in this investigation were subjected to gene target sequence (GTS) confirming that these two genotypes were *M. gallisepticum* wild strains. They revealed similarity with other strains with the designation (Rab-E 1-08 and Eis-5-C-10) characterized by Khalifa *et al*.[8] and Eissa *et al*.[28]. The sequence analysis of the *mgc2* genetic region was previously identified in *M. gallisepticum* isolated from tracheal swabs of commercial poultry in South Africa provinces[30].

The MIC test of the commonly used antimicrobial agents in the poultry industry was performed on the two sequenced isolates (RAG-1-MG-CK-Eg017 and RAG-2-MG-CK-EG 017). The results revealed the sensitivity of the first strain (RAG-1-MG-CK-Eg017) to tilmicosin, tiamulin and spiramycin, while the second strain (RAG-2-MG-CK-EG 017) showed sensitivity to tiamulin, spiramycin and tilmicosin. Similarly, Amer *et al*.[10] demonstrated that tilmicosin was effective in the treatment and limitation of *M. gallisepticum* infection in tested chickens. However, Jordan and Horrocks[31] proved that tilmicosin had a slightly lower MIC on *M. gallisepticum* than tylosin at both the initial and final readings. El-Aziz *et al*.[3] found that aivlosin, lincomycin: Spectinomycin (1:2), tylosin, tiamulin, enrofloxacin and lincomycin were commonly active against *M. gallisepticum*. Additionally, these results showed that lincomycin and doxycycline had limited activities against the examined strains of *M. gallisepticum*. In contrast, a previous study investigated an excellent effect of doxycycline against *M. gallisepticum* strain S6 recovered from chicken *in vitro*[14].

This investigation was indicated the requirement of checking the poultry farms for avian mycoplasmosis by SPA, ELISA and PCR, as well as application of MIC assay to decide the most effective antimicrobial agents. Further studies should be devoted to prevent and control mycoplasma in all stages of the poultry industry production chain and avoid economic losses in Egypt.

CONCLUSION

In conclusion, this study demonstrated that application of classical serological techniques and modern molecular techniques were fundamental tools for precise monitoring of *M. gallisepticum* infection in broilers under field conditions and confirm the importance of periodical sequence analysis to evaluate the epidemiological status of *M. gallisepticum* infection in the certain country. Additionally, the *in vitro* effectiveness of some antimicrobial agents used in the veterinary field on *M. gallisepticum* was evaluated. Lastly, more studies are required for controlling of avian mycoplasmosis in Egypt.

SIGNIFICANCE STATEMENT

This study was performed to explore the prevalence of *Mycoplasma gallisepticum* in diseased broilers and determination of its sensitivity to the most commonly used anti-mycoplasma drugs through the minimal inhibitory concentration assay. *Mycoplasma gallisepticum* (MG) is one of the causative agent related to economic losses in the Egyptian poultry industry, thus its detection and trials for treatment and controlling is necessary. This study will help the researchers to know the effective methods for precise monitoring of *Mycoplasma gallisepticum* infection in the poultry farms and its in vitro susceptibility to some antibiotics.

REFERENCES

1. Ley, D.H., 2003. Mycoplasma Gallisepticum Infection. In: Diseases of Poultry, Saif, Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald and D.E. Swayne (Eds.). 11th Eds., Iowa State Press, Ames, IA., pp: 722-744.
2. OIE., 2018. OIE world organization for animal health. Office International des Epizooties (OIE), Paris. http://www.oie.int/eng/normes/mmanual/A_00104.html
3. El-Aziz, N.K.A., I.E. Eldesoky, A.M. Ammar, S.I. Eissa and Y.H. Mohamed, 2014. Molecular studies on *M. gallisepticum* and avian pathogenic *E. coli* induced infections in broilers. Eur. J. Vet. Med., Vol. 4.
4. Liu, T., M. Garcia, S. Levisohn, D. Yogev and S.H. Kleven, 2001. Molecular variability of the adhesin-encoding gene *pvpA* among *Mycoplasma gallisepticum* strains and its application in diagnosis. J. Clin. Microbiol., 39: 1882-1888.
5. Ali, M.Z., M.M. Rahman and S. Sultana, 2015. Seroprevalence of *Mycoplasma gallisepticum* antibody by ELISA and serum plate agglutination test of laying chicken. Vet. World, 8: 9-14.
6. Khalifa, K.A., E.S. Abdelrahim, M. Badwi and A.M. Mohamed, 2013. Isolation and molecular characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chickens in Sudan. J. Vet. Med., Vol. 2013. 10.1155/2013/208026.
7. El-Kady, M.F., I.R. Abd Elhafeez, S.I. Eissa, A.M. Ibraheem and E.M. Hassan, 2013. Role of some live vaccines in activation of *Mycoplasma gallisepticum* infection in upper respiratory system of chickens. Ph.D. Thesis, Beni Suef University, Egypt.
8. Khalifa, R., S. Eissa, M. El-Hariri and M. Refai, 2014. Sequencing analysis of *Mycoplasma gallisepticum* wild strains in vaccinated chicken breeder flocks. J. Mol. Microbiol. Biotechnol., 24: 98-104.
9. Rasoulinezhad, S., M.H. Bozorgmehrifard, H. Hosseini, N. Sheikhi and S. Charkhkar, 2017. Molecular detection and phylogenetic analysis of *Mycoplasma gallisepticum* from backyard and commercial turkey flocks in Iran. Vet. Res. Forum, 8: 293-298.

10. Amer, M.M., G.A. Zohair, K.M. El-Bayomi and Z.M.A. Girh, 2012. Effect of tilmicosin in control of mycoplasmosis in broiler chickens from infected breeders using elisa test for evaluation. J. Am. Sci., 8: 696-700.
11. Grozner, D., Z. Kreizinger, K.M. Sulyok, Z. Ronai and V. Hrivnak *et al*., 2016. Antibiotic susceptibility profiles of *Mycoplasma* sp. 1220 strains isolated from geese in Hungary. BMC Vet. Res., Vol. 12. 10.1186/s12917-016-0799-0.
12. Zhang, N., X. Gu, X. Ye, X. Wu and B. Zhang *et al*., 2016. The PK/PD interactions of doxycycline against *Mycoplasma gallisepticum*. Front. Microbiol., Vol. 7. 10.3389/fmicb.2016.00653.
13. Frey, M.L., R.P. Hanson and D.P. Andrson, 1968. A medium for the isolation of avian mycoplasmas. Am. J. Vet. Res., 29: 2163-2171.
14. Erno, H. and L. Stipkovits, 1973. Bovine mycoplasmas: Cultural and biochemical studies. II. Acta Vet. Scand., 14: 450-463.
15. OIE., 2008. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE World Organization for Animal Health, Paris.
16. Garcia, M., N. Ikuta, S. Levisohn and S.H. Kleven, 2005. Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. Avian Dis., 49: 125-132.
17. Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.
18. Kumar, K.G., S. Ponsuksili, K. Schellander and K. Wimmers, 2004. Molecular cloning and sequencing of porcine C_5 gene and its association with immunological traits. Immunogenetics, 55: 811-817.
19. Lysnyansky, I., M. Garcia and S. Levisohn, 2005. Use of mgc2-polymerase chain reaction-restriction fragment length polymorphism for rapid differentiation between field isolates and vaccine strains of *Mycoplasma gallisepticum* in Israel. Avian Dis., 49: 238-245.
20. Tanner, A.C. and C.C. Wu, 1992. Adaptation of the Sensititre broth microdilution technique to antimicrobial susceptibility testing of *Mycoplasma gallisepticum*. Avian Dis., 36: 714-717.
21. Osman, K.M., M.M. Aly, Z.M.S. Amin and B.S. Hasan, 2009. *Mycoplasma gallisepticum*: An emerging challenge to the poultry industry in Egypt. Rev. Scient. Tech., 28: 1015-1023.
22. Ferguson-Noel, N., 2013. Mycoplasmosis. In: Diseases of Poultry, Swayne, D.E., J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez and V.L. Nair (Eds.). 13th Edn., Blackwell-Wiley Publishing, Ames, IA., pp: 875-876.
23. Dardeer, M.A., E.E. Abd Elaziz and A.A. Hanaa, 2006. Study of antigenic and genetic vaiability of *Mycoplasma gallisepticum* strains isolated from SASSO chicken breed in Sharkia and Dakahlia governorates, Egypt. Proceedings of the 12th Scientific Congress, (SC'06), Faculty of Veterinary Medicine, Assiut University, Egypt, pp: 601-608.
24. Abd El-Ghany, W.A., 2008. Diagnostic investigation on *Mycoplasma gallisepticum* infections in different Egyptian breeder and broiler chicken flocks. J. Egyp. Vet. Med. Assoc., 68: 29-45.
25. Feizi, A., H. Nikpiran, P. Bijanzad, A.R.J. Moggadam and H. Hosseini, 2013. Comparative evaluation of serological test in diagnosis of *Mycoplasma gallisepticum* infection in Iranian north-west rural poultry. Adv. Biores., 4: 50-53.
26. Perrin, G. and G. Bennejean, 1982. Le depistage of infection avian *Mycoplasma bullitin* aviaire. Bull. Lab. Ser. Vet., 5: 13-29.
27. Armour, N.K., V.A. Laibinis, S.R. Collett and N. Ferguson-Noel, 2013. The development and application of a *Mycoplasma gallisepticum* sequence database. Avian Pathol., 42: 408-415.
28. Eissa, S.L., A.M. Hassan, A.M. Metwally, Y.M. Hashem and E.E. Abd El-Aziz, 2011. Molecular characterization of *Mycoplasma gallisepticum* isolated from Chicken and Turkey. Vet. Med. J. Giza, 59: 183-195.
29. Moretti, S.A., C.E. Boucher and R.R. Bragg, 2013. Molecular characterisation of *Mycoplasma gallisepticum* genotypes from chickens in Zimbabwe and South Africa. South Afr. J. Sci., 109: 1-4.
30. Ferguson, N.M., D. Hepp, S. Sun, N. Ikuta, S. Levisohn, S.H. Kleven and M. Garcia, 2005. Use of molecular diversity of *Mycoplasma gallisepticum* by Gene-Targeted Sequencing (GTS) and Random Amplified Polymorphic DNA (RAPD) analysis for epidemiological studies. Microbiology, 151: 1883-1893.
31. Jordan, F.T. and B.K. Horrocks, 1996. The minimum inhibitory concentration of tilmicosin and tylosin for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* and a comparison of their efficacy in the control of *Mycoplasma gallisepticum* infection in broiler chicks. Avian Dis., 40: 326-334.