Mycoplasma gallisepticum: a devastating organism for the poultry industry in Egypt

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ABSTRACT *Mycoplasma gallisepticum* (**MG**) is a worldwide ruined bacteria affecting different avian species, causing severe economic losses. Consequently, the current research sought to detect the incidence of MG among different commercial broiler, layer chickens and turkey farms, and environmental litter samples in different Egyptian governorates (Damietta, Giza, El-Qalyobia, El-Sharqia, and El-Behera) from January 2019 to December 2020. Four hundred samples (infraorbital sinus aspirates, tracheal swabs, serum from diseased birds, and organ samples; lung tissues, air sacs and tracheal bifurcation from freshly dead birds), and environmental samples (litter) were collected for MG isolation. Samples were subjected to phenotypic and molecular identification. Positive bacteriological samples were subjected for molecular identification using polymerase chain reaction (**PCR**) test to detect MG, then sequencing for PCR amplicon of *mgc*2 gene. Out of 332 samples subjected for bacteriological examination, 206 were bacteriologically positive for MG with an incidence of 62%. The highest incidence of MG was detected in turkey farms at a rate of 83%, followed by broiler chicken farms, layer chicken farms and litter samples at a percentage of 70, 40, and 40, respectively. The highest prevalence of MG in chickens and turkey was recorded during the winter and autumn seasons. Molecular identification of MG isolates revealed that 85% of isolates were positive for *mgc*2 gene using PCR. The Four sequenced strains in this study are closely related and placed in one group with the vaccine strain 6/85 and ts11 strain.

Key words: chickens, chronic respiratory disease, infectious sinusitis, *Mycoplasma gallisepticum*, *Mgc2* gene

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INTRODUCTION

The Egyptian poultry industry is a lucrative investment opportunity since the domestic market for poultry meat consumption is large and has significant growth potential due to population increase and per capita consumption growth, driven by the country's improving economy (Shatokhin et al., 2017; Abdelnour et al., 2020a,b; Sheiha et al., 2020). *Mycoplasma* is a major pathogen that threatens the poultry industry and causes severe economic losses worldwide (Eissa et al., 2014; Marouf et al., 2020; Yadav et al., 2021).

Mycoplasmas are small prokaryotes that lack a cell wall, but they are surrounded by 3 layers of the plasma membrane, 300 to 800 nm in diameter. It can be detected in humans and animals, with an optimum growth temperature of 37°C with the ability to replicate outside of host cells (Brown et al., 2007; Abdel Halium et al., 2019; Abo Elyazeed et al., 2020; Sawicka et al., 2020). *Mycoplasma gallisepticum* is the most virulent and economically significant bacterial respiratory infection of poultry. The Office International des Epizootics

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(**OIE**) has declared the infection with MG as a notifiable disease (OIE, 2004). MG is a serious problem in the poultry field worldwide, causing chronic respiratory disease (**CRD**) in the broiler, layer, breeder flocks, and infectious sinusitis in turkeys. It also harms egg production (Eid et al., 2019; Lysnyansky et al., 2005). In addition, MG infection also exaggerates viral infections and secondary bacterial complications and adversely affects the efficacy of avian viral vaccines (Fathy et al., 2017). Diseased chickens with CRD show signs of respiratory symptoms like sneezing, rales, coughing, conjunctivitis, lacrimation, exudation from the nostrils, increased carcass condemnation and embryonic mortality, and reduced egg production, hatchability, feed conversion rate, and weight gain (Marouf et al., 2020).

Environmental samples constitute a continuous source for MG infection in poultry houses; strict environmental hygienic measures should be adopted to overcome this problem (Marois et al., 2002). Diagnosis of avian mycoplasmosis based on isolation, identification, antibodies detection, and polymerase chain reaction (**PCR**). Culturing pathogenic avian mycoplasmas organisms is difficult, slow-growing, relatively fastidious, and might require up to 3 wk for detectable growth. Therefore, the rapid serum plate agglutination test (**SPA**) and hemagglutination inhibition test (**HI**) tests have been used routinely. Because the SPA test is rapid, relatively less costly, and sensitive, it has been widely used as a primitive screening test for serological monitoring of poultry flocks (Kleven et al., 1998). Problems of low sensitivity, cross-reactions, and nonspecific reactions were encountered with rapid SPA and HI tests (Ewing et al., 1996; Hemeg et al., 2020). Molecular biology techniques such as PCR and Random amplified polymorphic DNA (**RAPD**) or the Arbitrarily PCR (**AP-PCR**) were applied for detection and identification of Mycoplasmas (Fan et al., 1995). This study aimed to repertoire the mycoplasmal picture of chicken and turkey flocks in many Egyptian governorates. The study shows MG at the phenotypic and genotypic level and environmental shedding, facilitating the vaccine used decision in farms.

MATERIALS AND METHODS

Animal Ethics

All bird samples and related procedures were ethically approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Cairo University, Egypt. (Vet.CU-IACUC) with approval number (Vet.CU-10102019093).

Samples Collection

Investigated broiler and layer farms suffered from respiratory manifestations and drop in egg production in layer farms while turkey farms suffered from infectious sinusitis. All investigated farms were reared birds in a deep litter system.

Four hundred samples of diseased birds and freshly dead (broiler and layer chickens suffered from CRD and complicated chronic respiratory disease [**CCRD**] as well as diseased turkey suffered from swelling in infraorbital sinus in addition to litter samples) were collected at different ages and different seasons from Damietta, Giza, El-Qalyobia, El-Sharqia and El-Behera Governorates between January 2019 and December 2020. The number and type of collected samples are summarized in Table 1.

Microbiological Studies

The samples were subjected to bacterial isolation and identification by samples inoculated into Frey's broth and incubated at 37°C with 5 to 10% Co_2 and humidity for 48 h following the standard procedures of Sabry and Ahmed (1975). Then, a loopful from each incubated sample, were streaked on pleuropneumonia-like organisms (**PPLO**) agar by drop technique and then incubated at 37°C with 5 to 10% Co_2 with humidity for 10 to 14 d and examined under dissecting microscope for characteristic fried egg appearance of *Mycoplasma* colonies. Bio-typing of the purified colonies based on digitonin sensitivity test to distinguish between *Mycoplasma* and *Acholeplasma* isolates were adopted according to

Table 1. Sample's repertoire (type of samples, bird status, type, and number of examined of specimens).

Samples	Examined birds and environmental samples	Bird status	Type of specimens		No. of examined specimens
Broiler (100)	50	Diseased chicken	Blood (serum)		50
			Tracheal swab		50
	50	Dead chicken	Organs	Lung	50
				Tracheal bifurcation	50
				Air sacs	50
Layer (36)	18	Diseased chicken	Blood (serum)		18
			Tracheal swab		18
	18	Dead chicken	Organs	Lung	18
				Tracheal bifurcation	18
				Air sacs	18
Turkey(30)	30	Diseased	Infraorbital sinus aspirates		30
Environmental	10	Broiler farms	Litter		10
samples (30)	10	Layer farms	Litter		10
	10	Turkey	Litter		10
Total					400

Edward and Freundt (1973). Glucose fermentation test was performed according to Ernq and Stipkovitls (1972), arginine hydrolysis, film and spot formation tests were performed. The growth inhibition test was formed to identify mycoplasmas isolate depending on serology. The serum samples were analyzed by serum agglutination plate based on Sabry and Ahmed (1975).

To develop a pure culture, one fried egg-shaped colony was chosen and inserted into a broth medium, along with the agar block. Purified isolates were kept at −20°C in the form of agar blocks.

Molecular Identification

DNA from isolated colonies was purified using QI Aquick PCR Product extraction kit. (Qiagen Inc. Valencia, CA). Oligonucleotide primer encoding *mgc*2 gene (Mgc2 F: 3'CGC AAT TTG GTC CTA ATC CCC AAC A'5; Mgc2 R: 5' TAA ACC CAC CTC CAG CTT TAT TTC C'3) of 300 bp was specific for MG (Lysnyansky et al., 2005). The reaction mixture (total volume of 50 μL) was 5 μL of 10X PCR reaction buffer (Promega), 5 μL 1 m MdNTP mix (Sigma), 4 μL of 25 Mm $Mgcl_2$, 5 μL DNA *Taq* polymerase, 1 μL containing 5 Mm of each primer, 5 μL of sample DNA. Then 0.25 mL Go-*Taq* polymerase (Promega) was added and the mixture was completed by sterile distilled water. The PCR protocol was repeated 40 cycles as listed by Ferguson et al. 2005; (Denaturation 94°C for 30 s, Annealing 55°C for 30 s, Extension 72°C for 1 min and Final extension 72°C for 5 min). Aliquots of amplified products (10 μL) were electrophoresed through 1% agarose gel and DNA was visualized by Ultraviolet transilluminator after ethidium bromide staining then photographed (Sambrook et al., 1989) using a digital camera (Acer CR-5130, Nanjing, China).

Sequence Analysis

On an applied biosystems 3130 automated DNA Sequencer, a purified RT-PCR product was sequenced in both forward and reverse directions (ABI, 3130). Using a PerkinElmer/Applied Biosystems, Foster City, CA, Bigdye Terminator V3.1 cycle sequencing kit (Cat. No. 4336817).

To determine sequence identity to Gen Bank accessions, a BLAST analysis (Basic Local Alignment Search Tool) was used first (Altschul et al., 1990). The sequence reaction was carried out according to the manufacturer's instructions.

Phylogenetic Analysis

The CLUSTAL W multiple sequence alignment program, version 1.83 of Meg Align module of Laser gene DNA Star software Pairwise, designed by Thompson et al. (1994), was used to compare sequences. To determine nucleotide and amino acid sequence similarities and linkages, the Meg Align module of the Laser gene DNA Star program was used to perform sequence alignments and phylogenetic analyses of the aligned sequences for the gene.

RESULTS

Clinical, Postmortem Investigations and Phenotypic Analysis

Investigated chicken farms suffered from respiratory manifestations such as conjunctivitis, cough, sneezing, gasping, rales, nasal discharge, ruffled feather, sleepy appearance, inappetence with general depression, and drop in egg production with the presence of eggshell abnormalities in layer flocks. Examined turkey farms suffered from infectious sinusitis with unilateral or bilateral swelling in infraorbital sinuses, conjunctivitis as seen in Figure 1 with nasal discharge, ruffled feathers, and general depression.

The postmortem examination of freshly dead birds revealed the presence of variable degree (foamy to caseous) air saculitis, pericarditis, perihepatitis, general serositis as seen in Figure 2. Out of 332, only 206 samples appeared fried egg appearance with depressed center colonies with cultivation ratio of (62%) as seen in Figure 3. Bio-typing profile reveals a ratio of 95% (196/206) were digitonin sensitive as seen in Figure 4, glucose positive, arginine negative and form Film and spot.

The incidence of MG infection between bacteriologically examined samples was 62%. The highest incidence was detected in turkey farms 83%, followed by chicken broiler farms 70%, then chicken layer arms 40%, and 40% from litter samples, as summarized in Table 2.

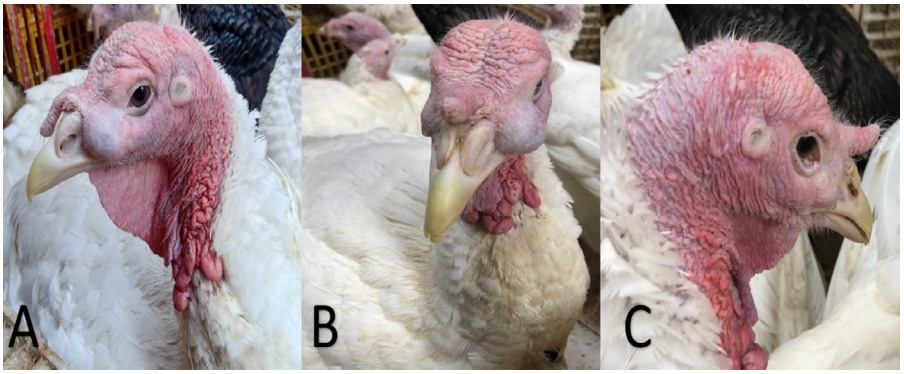

Figure 1. (A, B) Adult turkey showing unilateral swelling in infraorbital sinus, (C) adult turkey showing severe conjunctivitis.

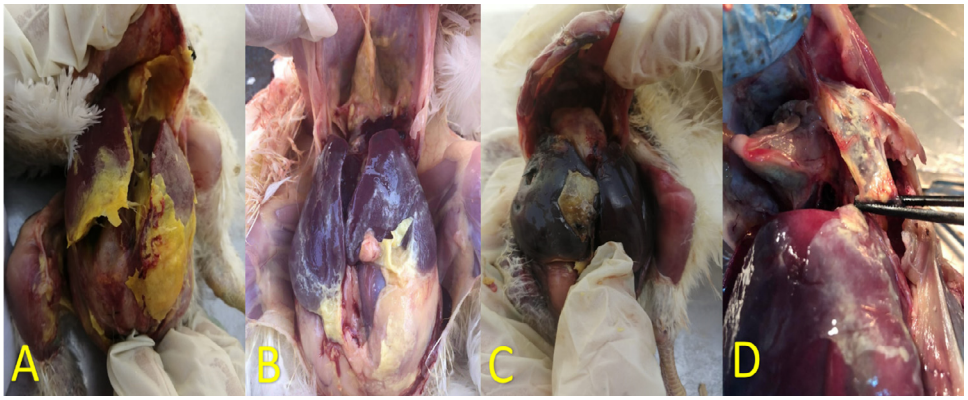

Figure 2. (A, B) Postmortem of freshly dead broiler chickens showing caseous pericarditis, perihepatitis and air saculitis. (C) Postmortem of freshly dead layer chicken showing air saculitis, pericarditis and perihepatitis (CCRD).

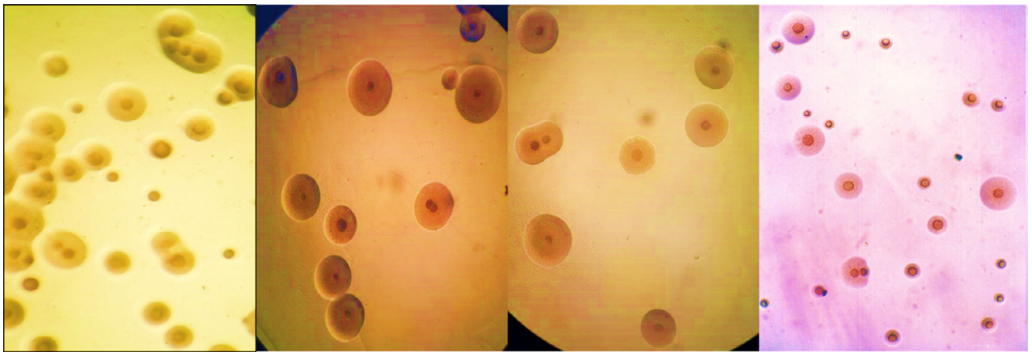

Figure 3. Microscopical appearance of *M. gallisepticum* with characteristic fried egg appearance with depressed center colonies.

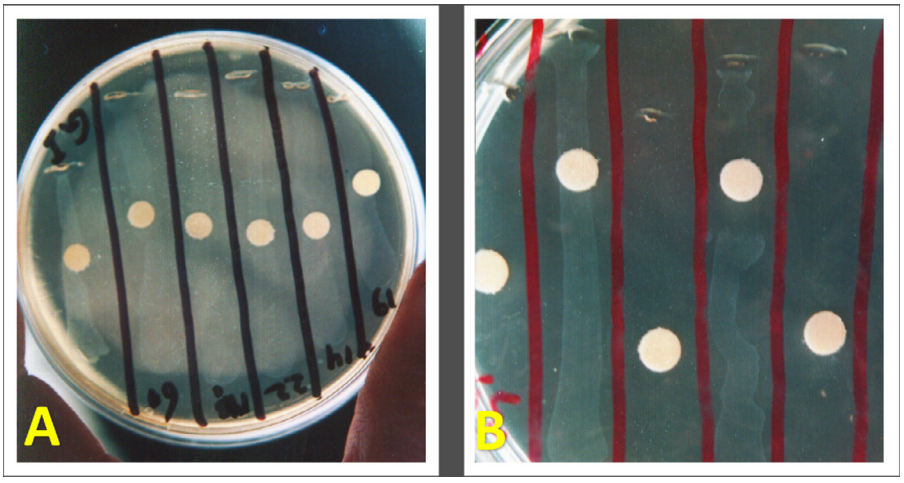

Figure 4. (A) Growth inhibition test against *M. gallisepticum* antiserum. (B) Digitonin sensitivity test.

Serological Analysis

At the examination, all serum samples were agglutinated at a ratio of 100% using a slide agglutination plate (**SAP**). The suspected colonies were serologically investigated using a growth inhibition test against specific MG antisera, which showed a ratio 95% (196/206), as seen in Figure 4.

Table 2. Incidence of *Mycoplasma gallisepticum* (MG) infection.

Type of sample	No. of bacteriologically examined samples	No. of MG positive samples	Incidence of positive samples
Broiler	200	140	70%
Layer	72	29	40%
Turkey	30	25	83%
Litter	30	12	40%
Total	332	206	62%

Molecular Analysis

PCR characterized 206 suspected colonies by using MG primer (*mgc*2 gene). One hundred and seventy-five colonies had amplified fragments at 300 bp with a ratio of 85%, as shown in Figure 5.

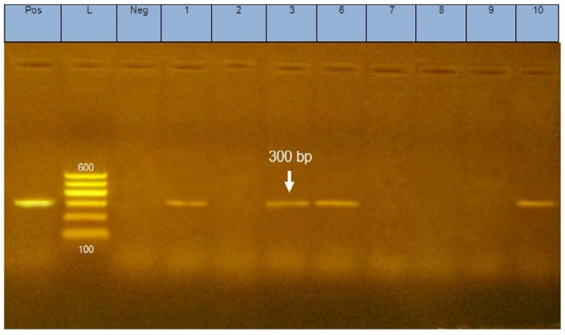

Figure 5. Agarose gel electrophoresis showing amplification fragments of 300 bp of MG (*mgc2* gene). Lanes 1,3,6,10 are field isolates of *M. gallisepticum*; positive control (*M. gallisepticum* S6) and negative control (*Escherichia coli*). L shows a 100−600 bp ladder.

DNA Sequencing Result

Sequencing of *mgc*2 gene was conducted in both directions and a consensus sequence of 300 bp was used for nucleotides and deduced amino acid analysis. The original sequence was trimmed to remove indefinite nucleotides. Sequences usually exist at the beginning of the sequencing reaction. Four *mgc*2 sequences were submitted to GenBank database where obtained the accession numbers; MG496039 (2032138 seq 1), MG356828 (2032742 seq1), MG356829 (2058726 seq1), and MG356830 (2058734 seq1). Identification of homologies between nucleotide and amino acid sequences of the Egyptian MG strains and other strains published on GenBank was made using BLAST 2.0 and PSI-BLAST search programs (National Center for Biotechnology Information "NCBI" http://www.ncbi.nlm.nih.gov/), respectively. The obtained nucleotide sequences and the deduced amino acid of Egyptian MG strains and other strains published on GenBank were made using the Bio-Edit sequence alignment editor, ClustalW software for multiple sequence alignment, Clusta lV and Meg Align, DNA STAR, Laser gene, Version 7.1.0. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by the Neighbor-joining method based on ClustalW. MegAlign calculated sequence divergence and identity percent. The histological character of the mgc2 protein sequence was identified by Protean (DNASTAR, Lasergene, Version 7.1.0, Germany) by measuring the antigenicity index.

The four samples showed 73.1% maximum identity to the MG - strain - F - Mgc2 strain (Accession no. KJ364633), 76.7% maximum identity to the MG − strain − Eis 10 − 17 (Accession no. KY421065) and MG − strain - KS2 (Accession no. AY556293), 76.4% maximum identity to the MG − strain − R − Mgc2 (Accession no. AY556228), 77.4 % maximum identity to the MG − strain − ts - 11 − Mgc2 (Accession no. JQ770175) and 99.3% maximum identity to the MG − strain − VH1 − Mgc2 (Accession no. JX981926), MG − strain − MGS 1169(Accession no. KP300759) and MG − strain − 6 - 85(Accession no. KP318741).

The four sequenced isolates in this study are closely related and placed in one group with the vaccine strain 6/85 and ts11 strain. They are distinct from other field isolates from Egypt and other countries from Ks2 strain and far from the recently identified isolates from the Middle East from R strain and the original vaccine strain F as represented in Figures 6 and 7.

Incidence of MG in Examined Samples

The incidence of MG isolation is summarized in Tables 2 and 3. Following all diagnostic technique in the study, 206 mycoplasmas showed fried egg appearance of MG while 175 isolates were confirmed by PCR (85%).

Through tracking complaints in farms, most of the samples were collected in winter (60%) and autumn (25%), followed by spring (10%) and finally summer (5%). MG was identified in Damietta, Giza, El-Sharqia, El-Behera and El-Qalyobia Governorates with ratio 41.2 (85/206), 40 (72/206), 10.6 (22/206), 7.2 (15/206), and 5.8% (12/206), respectively.

The highest isolation percentage of sample types was 83, 79.4, 72, 67.4, 40, and 29.4% from infraorbital sinus

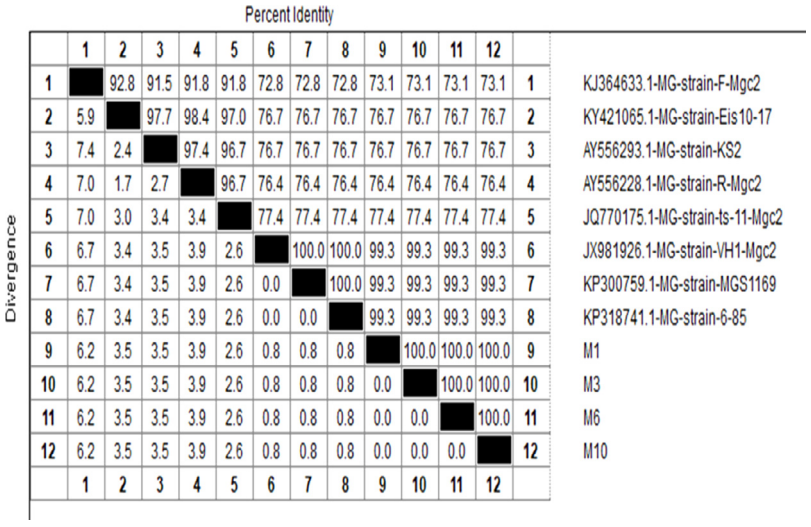

Figure 6. Sequence distance between the samples.

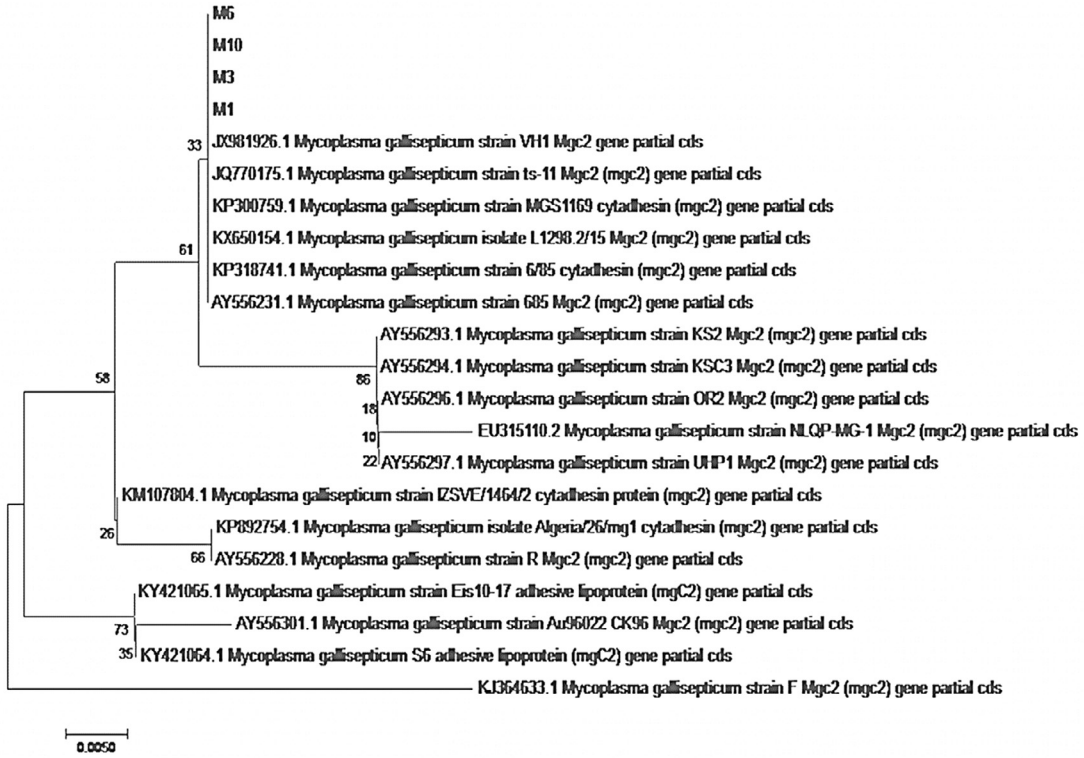

Figure 7. Phylogenetic tree of the four sequenced samples.

aspirates, lung, air sacs, tracheal bifurcation, litter samples, and tracheal swabs, respectively.

DISCUSSION

The poultry business is one of Egypt's most important agricultural industries, providing a significant portion of the country's animal protein supply (white meats and eggs) (Salem and Attia, 2021). The chicken industry is tied to other industries such as animal feed, medication, and veterinary inputs and provides animal protein for human use. Respiratory diseases represent a great threat to the poultry industry worldwide (Setta et al., 2018; El-Naggar et al., 2021). MG is the most pathogenic species within the genus *Mycoplasma* of the family *Mycoplasmataceae* that consider poultry devastating (Evans and Leigh 2008; Erfan and Marouf, 2019; Prabhu et al., 2021). The present investigation shows the *Mycoplasma* incidence in different Egyptian governorates based on classical and modern techniques.

Our results showed that the investigated chicken flocks showed variable signs of respiratory manifestations and that postmortem examination revealed variable degree air saculitis, pericarditis, perihepatitis, and serositis. Similar findings were recorded by Saif et al. (2003), Bharathi et al. (2018) and Emam et al. (2020). Our investigations showed that diseased turkeys suffered from unilateral or bilateral swelling in the infraorbital sinuses with conjunctivitis, mild respiratory manifestation, and general depression. Similar findings were recorded by Prabhu et al. (2021) in turkeys infected with MG in India. The Biotyping profile of MG reveals a ratio of 95% (digitonin sensitive, glucose positive, arginine negative and form Film and spot tests as indicated in Roussan et al. (2008). Serological tests are monitoring methods for MG infection in a flock. Serological analysis in work by SAP was positive with a ratio of 100%. These tests showed many cross-reactivities (Abdelmoumen and Roy, 1995; Eljakee et al., 2011; Osman et al., 2014; Abu-Seida et al., 2015). The suspected colonies were

Table 3. Incidence of *Mycoplasma gallisepticum* (MG) isolation.

Items	Examined samples Number = 400 (68 sera + 332 samples for bacterial isolation)	Positive		Negative	
		Number	%	Number	%
SPA test	Serum (68)	68	100	0	0
Bacterial isolation	Tracheal swabs (68)	20	29.4	48	70.6
	Lung (68)	54	79.4	14	20.6
	Trachea bifurcation (68)	46	67.4	22	32.6
	Air sacs (68)	49	72	19	28
	Infraorbital sinus aspirates (30)	25	83	5	17
	Litter samples (30)	12	40	18	60
Total	(332) samples used for bacterial isolation	206	62	126	38

serologically examined by growth inhibition test against specific MG antisera and revealed a ratio of 95%, as mentioned by Osman et al. (2009).

From our results, the highest isolation percentage was 83% from infraorbital sinus aspirates, 79.4% from lung, 72% from air sacs, 67.4% from tracheal bifurcation, 40% from litter samples and 29.4% from tracheal swabs. Also, Mohamed (1997), Reda and El-Samie (2012), Emam et al. (2020) showed that the highest isolation percentage was from air sacs as 15.7, 23.3, and 21.5%, respectively.

From our finding, the lowest isolation percentage was from the tracheal swabs (29.4%). On the other hand, El-Jakee et al. (2019) obtained 14 *Mycoplasma* isolates from tracheal swabs collected from 12 broiler breeder flocks. From our results, out of 30 litter samples from different sources (broiler, layer chickens and turkey litter), only 12 samples were positive for MG infection. We concluded that the litter is a continuous source of *Mycoplasma* infection, so it should be treated with appropriate disinfectants before getting rid of it to limit the spread of *Mycoplasma*. This finding agrees with Marois et al. (2002) reported as they were isolated and molecularly detected MG in environmental samples collected from turkey farms. From our results, the widespread prevalence of MG in Egypt increases in the winter and autumn seasons. It can be related to that the fall season is thought to favor microbial multiplication and excessive ammonia levels, poor ventilation accompanied with expanded poultry stocking capacity, and create a favorable environment for respiratory pathogens infection (Hassan et al., 2016). From our findings, the incidence of MG infection was 62%. The highest incidence was noticed in turkey farms, 83%, followed by chicken broiler farms, 70%, then chicken layer arms 40%, and 40% from litter samples. The incidence profiles of the study, as mentioned in the results, is closely nearly to the conclusion of Bharathi et al. (2018), Erfan and Marouf (2019), Emam et al. (2020), Marouf et al. (2020). On the other hand, the layer flocks are more prevalent in Osman et al. (2009) study.

The conventional, traditional methods for MG isolation and identification are laborious, time-consuming, less sensitive, and fail to detect *Mycoplasma* species from treated birds (Emam et al., 2020). Meanwhile, the PCR technique is a quick, sensitive, and accurate method for detecting MG from suspected cases (Gondal et al., 2015). From our investigations, 175 colonies had amplified fragments at 300 bp by PCR based on *mgc2* gene as shown in Figures 6 and 7 with a ratio of 85% that nearly agree with those recorded by Heleili et al. (2012) and Bayatzadeh et al. (2011), Emam et al., 2020 who found the incidence of *Mycoplasma* was 60.33, 60, and 87.5%, respectively. The PCR has various advantages. However, it is constrained by the formation of contamination because of improper sample handling, which results in false-positive results (OIE. 2000). As a result, traditional cultural approaches should be used in parallel with PCR (Emam et al., 2020).

Sequencing of *mgc2* gene for 4 isolates in this study are closely related to each other and placed in one group with the vaccine strain 6/85 and ts11 strain. These results are like those reported by Eissa et al., (2014), who stated mutations located in the *mgc2* region led to changes in the antigenicity indices in this region of *mgc2* protein compared to other published MG strains. Efforts should be intensified to reduce the negative impacts of mycoplasmosis in the poultry industry by adopting biosafety measures and effective vaccinations in the breeder flocks to minimize the vertical transmission of the disease (El-Naggar et al., 2021). It is also supposed to use some safe products such as herbal extracts (Abou-Kassem et al., 2021a), essential oil (Abd El-Hack et al., 2021a; Alagawany et al., 2021a; El-Tarabily et al., 2021), amino acids (Abou-Kassem et al., 2021b; Arif et al., 2021), phytogenic products (Abd El-Hack et al., 2021b,c; El-Shall et al., 2021; Reda et al., 2021), enzymes (Llamas-Moya et al., 2019), bioactive peptides (El-Saadony et al., 2021a,b), probiotics (Alagawany et al., 2021b; El-Saadony et al., 2021c), plant bioactive compounds (Abd El-Hack et al., 2021d; El-Saadony et al., 2021d), and prebiotics (Abd El-Hack et al., 2021e; Yaqoob et al., 2021), and biological synthesized nanoparticles (Abd El-Ghany et al., 2021; Abd El-Hack et al., 2021f) to raise the general health status of birds and improve their resistance to different diseases including mycoplasmosis.

CONCLUSIONS

Mycoplasma gallisepticum is a severe pathogen that threatens the poultry industry. The results confirmed the importance of applying the theory of prevention programs, hygienic disposal of farm wastes, effective, safe treatments, and vaccination for reducing MG infection in Egypt and saving this industry. The conventional isolation method for MG diagnosis is still the gold standard, even though it is time-intensive, and PCR is more rapid and accurate than conventional methods. Sequencing analysis suggested the use of strain 6/85 and ts11 strain in vaccines application.

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DISCLOSURES

The authors declare no conflict of interests.

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