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Phenotypic and Molecular Detection of *Mycoplasma gallisepticum* in Broiler and Layer Chickens in Some Egyptian Governorates

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Abstract

Mycoplasma gallisepticum (MG) is a major avian pathogen revealing financial losses in poultry industry around the world. The current research aimed to study the bacteriological, molecular detection and sequencing of MG recovered from cases with chronic respiratory disease (CRD) from broiler and layer chicken farms in some Egyptian governorates. Therefore, a total of 125 samples were collected from (25) flock/governorate, representing (15) broiler chicken and (10) laying chicken farms from five Egyptian governorates (Giza, Al-Qalyubia, AL-Dakahlia, Al-Faiyum, AL-Sharqia) from March 2020 till January 2022. The isolates were characterized by using conventional methods and molecularly identified by using (16S rRNA), (*mgc2*) specific primers for MG. The bacteriological isolation showed that 80.8% (101/125) samples appeared as fried egg colonies on PPLO agar while the rest 24 samples were negative. Only 88 out of 101 (87.1%) bacteriologically positive samples were positive by conventional PCR. Gene target sequencing (GTS) was carried out on one MG isolate with positive PCR *mgc2* specific gene. The incidence of MG increases in winter and autumn compared to summer and spring. The sequenced isolate of *mgc2* gene is grouped with field strain isolated from Egyptian chickens with 100% identity. The obtained findings indicated that the *mgc2* gene could discriminate between MG field type and vaccinal F-strain, MG remains a serious avian pathogen and the study recommend a periodical molecular monitoring of MG in poultry flock with sequence analysis of the circulating strains and further investigations should be applied to detect most suitable control and preventive measures to solve such problem.

KEYWORDS
Mycoplasma gallisepticum, *mgc2*,16S rRNA, PCR, Sequence analysis.

INTRODUCTION

In the poultry industry, respiratory tract diseases are of critical importance (Chaidez-Ibarra *et al*., 2022; Yehia *et al*., 2023). *Mycoplasma* is a major disease that poses a serious threat to the poultry industry and results in significant financial losses around the world (Marouf *et al*., 2020; Yadav *et al*., 2021; El-Naggar *et al*., 2022). *Mycoplasma* costs the poultry sector a lot of money because it reduces production by 10–20 % and increases embryonic mortality by 5–10 %, in addition to the costs of prevention and control (Nascimento *et al*., 2005; Jaÿ *et al*., 2021; Qoraa *et al*., 2023). *Mycoplasma* infection could be spread either by horizontal or vertical routes (Matucci *et al*., 2020), and can affect chickens and turkeys at all ages, but young birds are more susceptible (Ali *et al*., 2015). Several types of pathogenic *Mycoplasma*s could attack avian species while, *Mycoplasma gallisepticum* (MG) is the most serious one and it has been classified as a notifiable disease by the Office International des Epizootics (OIE) (OIE, 2004; Chaidez-Ibarra, 2022). *Mycoplasma gallisepticum* infections cause chronic respiratory disease (CRD) in chickens and infectious sinusitis (IS) in turkeys (Emam *et al*., 2020).

Mycoplasma gallisepticum induces a wide range of losses in layer, broiler, and breeder flocks (Gondal *et al*., 2013; Emam *et al*., 2020). Furthermore, MG infection enhances co-infections, as well as reducing the effectiveness of avian viral vaccinations (Fathy *et al*., 2017; Marouf *et al*., 2022). To effectively limit the infections with MG, it is necessary to identify affected birds to reduce the danger of infection spreading to healthy birds, as well as to prioritize care and control efforts in areas where MG is prominent as there were multiple reports of *Mycoplasma* seroprevalence in chicken farms around the world (Ali *et al*., 2015; Xue *et al*., 2017; Rehman *et al*., 2019). So, early detection and control of outbreak would be considerably assisted by fast and effective detection of avian pathogenic *Mycoplasma*s (Pang *et al*., 2002; Yadav *et al*., 2021). Clinical symptoms alone are insufficient to diagnose MG infection in poultry flocks (Bradbury *et al*., 2001). Thus, in most conditions, traditional bacteriological culture, serology, and molecular methods are used to diagnose MG infection (Nascimento *et al*., 2005; Sprygin *et al*., 2010; Yadav *et al*., 2021). However, culturing pathogenic avian *Mycoplasma*s organisms in Frey's/ pleuropneumonia-like organism broth or/and agar media is the gold standard for detecting MG but is laborious, slow-growing, and delicate, needing up to three weeks for significant growth and limited by the overgrowth problem of saprophytic *Mycoplasma*s and other microbial infections (Sprygin *et al*., 2010; Marouf *et al*., 2021). As a necessity, a quick, accurate, low-cost, and conclu-

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sive diagnostic approach for MG is needed, nucleic acid-based amplification techniques such as PCR procedures are generally accepted because they are faster and less expensive than culture methods (He *et al*., 2013; Tomar *et al*., 2017; Halium *et al*., 2019; Wu *et al*., 2019). However, it necessitates specialized laboratory facilities, experienced manpower, and a high cost for pathogen screening and detection, which limit its use in asset laboratories, primarily in developing world (Ahmed *et al*., 2015). Earlier attempts to detect MG from MS using PCR assays were focused primarily on the 16S rRNA gene (Garcia *et al*., 1996; Ben Abdelmoumen Mardassi *et al*., 2005) but the 16S rRNA gene's ubiquitous nature makes it difficult to distinguish between closely related *Mycoplasma* strains (Hong *et al*., 2004; Ben Abdelmoumen Mardassi *et al*., 2005). Current PCR methods target species specific areas such as (pvpA, gapA, MGA 0319, lipoprotein, *mgc2* and 16S-23S rRNA) for the detection of MG are commonly used (Yadav *et al*., 2021). Molecular techniques, on the other hand, should be used in combination with conventional serologic surveys and conventional cultures methods to identify *Mycoplasma* infections (Chaidez-Ibarra *et al*., 2022). Therefore, the current research was purposely designed to make a survey study on current situation of MG in layer and broiler chicken farms in some Egyptian governorates based on different *Mycoplasma* diagnostic techniques including culturing media PPLO, PCR techniques and sequencing to know evolution situation of circulating MG strains in Egypt.

MATERIALS AND METHODS

Ethical approval

This work is ethically approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University (Vet. CU. IACUC) with number Vet CU 2009 2022519.

Samples collection and area of study

The samples were collected from five Egyptian governorates (Giza, Al-Qalyubia, AL-Dakahlia, Al-Faiyum, AL-Sharqia). From each governorates samples were recovered from 25 flocks suffering from respiratory manifestation and un-vaccinated against *Mycoplasma*, representing (15) broiler and (10) layers chicken farms for the detection of MG infection during the period from January 2020 till March 2022. From each farm, pooled samples (tracheal bifurcation, lung, and air sacs) were collected from three freshly dead birds, and they were considered as a single unit/ sample under the research (one sample). Pooled samples from tracheal bifurcation, and lung were collected from recently diseased cases, whereas air sac specimens and caseous material were collected from chronic complicated cases. The number of specimens collected, and result of culture are listed in Table 1.

Isolation, culturing, and tentative identification

The pooled samples were inoculated into Frey's PPLO broth and incubated at 37°C with 5 to 10% CO_2 and humidity for 48 hours as described by Sabry and Ahmed (1975) usual protocols with addition of horse serum, yeast extract, and dextrose to the media, and addition of bacterial and fungal growth inhibitors (penicillin and thallium acetate following OIE (2018) recommendations. A loopful of each incubated sample was streaked on PPLO agar using the drop technique, and then incubated for 10 to 14 days at 37°C with 5 to 10% CO_2 and humidity (Edward and Freundt, 1973). The production of pellicle and changes in color from pink to yellow without any turbidity shows the growth of *Mycoplasmas* in broth medium but, *Mycoplasma* colonies in the shape of a fried egg or a nipple are commonly seen on solid medium under dissecting microscope (Kleven and Ferguson-Noel, 2008). To distinguish between *Mycoplasma* and Acholeplasma isolates, the digitonin sensitivity test was applied according to Freundt (1983). The glucose fermentation test and arginine hydrolysis were used to distinguish between various species of avian *Mycoplasma* (Ernø and Stipkovits, 1973).

Isolate purification and maintenance

To generate a pure culture, a single fried egg-shaped colony was selected, including the agar block, and transferred into a broth media and the purified isolates were stored in agar blocks at -20°C.

Molecular Identification

DNA extraction

All presumed positive samples from unvaccinated flocks with colonies like fried eggs undergo PCR testing for confirmatory testing. Samples with typical *Mycoplasma* colonies were their DNA extracted using a commercially available genome extraction gene direx (simply) kit, as per the guidelines provided by the manufacturer, DNA was gathered in sterile Eppendorf and kept at -20°C up to usage. We utilized two forward and reverse MG-specific primers to amplify a 185 base pair region of the 16S rRNA gene following Ghadimipour *et al*. (2018) methodology.

The sequence of 16S rRNA primer (F:5' AGCTAATCTGTAAAGTTGGTC 3'. (R: 5' GCTTCCTTGCGGTTAGCAAC 3), an underlying cycling condition of (initial denaturation at 95°C for 5 min, then 95°C for 45 second (denaturation) then annealing step at 53ºC for 30 sec then extension step at 72°C for 30 sec for 35 cycles, with the last extension step occurring at 72°C for 5 min.

Other specific primers to detect MG amplifying a 300 bp region of *mgc2* gene in accordance with the Lysnyansky *et al*. (2005) method, The sequence of *Mgc2* primer (F:5' CGC-AAT-TTG-GTC-CTA-ATC-CCC-AACA, (R: 5'TAA-ACC-CAC-CTC-CAG-CTT-TAT-TTC 3) an underlying cycling condition of (initial denaturation at 94°C for 5 min, then 94°C for 30 second (denaturation) then annealing step at 58ºC for 30 sec then extension step at 72°C for 30 sec for 35 cycles, with the last extension step occurring at 72°C for 7 min. A total volume of 25 µL was used for the amplifications, which included 3 µL of DNA template, 2µL of each primer (10 picomole), 12.5 µL of Taq DNA polymerase, and the remaining sterile distilled water. in the process of gel electrophoresis, amplified PCR products were run on a 1.5% agarose gel using Tris-boric acid-EDTA buffer pH 8.0 for 45 min at 1.7 volts and seen using an ultraviolet transilluminator.

MG Cyto-adhesion 2 gene (*Mgc2*) sequencing and phylogenetic analysis

One detected MG isolate were purified using QIA-quick PCR purification Kits (Qiagen Inc. Valencia CA), according to the manufacturer's instructions. Applied Biosystems (3130) automated DNA Sequencer/Perkin-Bigdye Elmer's Terminator V3.1 cycle sequencing kit, Cat. No. 4336817 was used to sequence purified products in both forward and reverse directions. To verify sequence identity with previously published sequences on gene banks, a BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) was first performed. The sequence was compared with other gene bank sequences by using the CLUSTAL W multiple sequence

alignment algorithm, version 1.83 of Meg Align module of Laser gene DNA Star software Pairwise, mentioned by Thompson *et al.* (2011). for phylogenetic analysis applying maximum likelihood, MEGA6 was applied. One selected strain was sequenced and submitted to GenBank with the accession number OQ129762 and compared to other local and worldwide MG sequences published on GenBank.

RESULTS

Clinical and postmortem examinations

Conjunctivitis, coughing, sneezing, tracheal rales, gasping, nasal discharge were respiratory signs found in the examined chicken farms with lethargic appearance, ruffled feathers, inappetence, depression, and decrease in egg production with the presence of abnormal shell quality in laying farms.

Freshly dead birds' PM examinations indicated varying degrees (foamy or caseous) air sacculitis, perihepatitis, pericarditis, and generalized serositis as seen in Figure 1.

Incidence of MG in the examined samples

The bacteriological investigation showed that out of 125 samples only 101 (80.8%) samples were positive for MG isolation and showed typical fried egg appearance (Fig. 1) on PPLO agar and were positive for *Mycoplasma* on digitonin test with a detection rate (100%) representing 68/101(67.3%) from broiler chickens and 33/101 (32.6%) from layer chickens. This result indicate to the isolation rate of MG was higher in broiler farms than layers.

The highest isolation percentage in broiler farms was detected in samples collected from Al Qalyubia governorates (100%), while the lowest percentage was detected in Al Sharqia governorates (80%). Regarding the layer farms, the highest isolation percentage was observed in Al Faiyum governorates (80%), while the lowest percentage was recorded in AL Dakahlia governorates (50%).

The detection rate using traditional bacteriological methods was 68/75 (90.65%) in broiler farms, this result from total broiler farms (75) and 33/50 (66%) in layers farms, this result from total layer farms (50), these positive samples were subjected for molecular identification using 16S rRNA and *mgc2* genes and the percentages of positive samples were 62/68 (91.1%) broiler farms, this result from total bacteriological positive broiler farms (68) and 26/33 (78.7%)-layer farms, this result from total bacteriological positive layer farms (33), *Mycoplasma gallisepticum* was glucose fermentation positive but arginine hydrolysis negative.

As seen in Figures 3 and 4, using MG specific primer (16s RNA and virulence gene *mgc2* gene), PCR was used to characterize 101 suspect colonies, 88/101 (87.1%) colonies had amplified fragments at 185 bp and 300 bp respectively were 62/88 (70.45%) positive from broiler farms and 26/88 (29.54%) from layers. which was reported in the governorates of Giza, Al-Qalyubia, AL Dakahlia, Al-Faiyum and Al-Sharqia with ratios of (17/101), (20/101), (17/101), (19/101), and (15/101), respectively.

Out of the 101 isolates, 88 were confirmed as MG by conventional PCR, while the highest ratio seen in broiler in Al-Qalyubia and in layer in Al- Faiyum according to Table 1.

During this study, the spread of respiratory disease infection and the isolation rate of MG was higher in the winter (67) and the autumn (20) in contrast to the summer (4) and spring (9).

Sequencing Findings

Both directions of the *mgc2* gene were sequenced, and a consensus sequence of 300 base pairs was used for deduced amino

Table 1. Number of examined samples and results of MG bacteriological identification and molecular detection in layer and broiler chicken farms in different Egyptian governorates.

Governorates	Total examined farm (No.)	Type		Positive culture (No.)		Positive culture (%)		Positive PCR (No.)		Positive PCR (%)	
		Broiler	Layer	Broiler	Layer	Broiler	Layer	Broiler	Layer	Broiler	Layer
Giza	25	15	10	13	7	86.6	70	12	5	92.3	71.4
Al Qalyubia	25	15	10	15	7	100	70	14	6	93.3	85.7
AL Dakahlia	25	15	10	14	5	93.3	50	13	4	92.8	80
Al- Faiyum	25	15	10	14	8	93.3	80	12	7	85.7	87.5
AL-Sharqia	25	15	10	12	6	80	60	11	4	91.6	66.6
Total	125	75	50	68	33	90.6	66	62	26	91.1	78.7

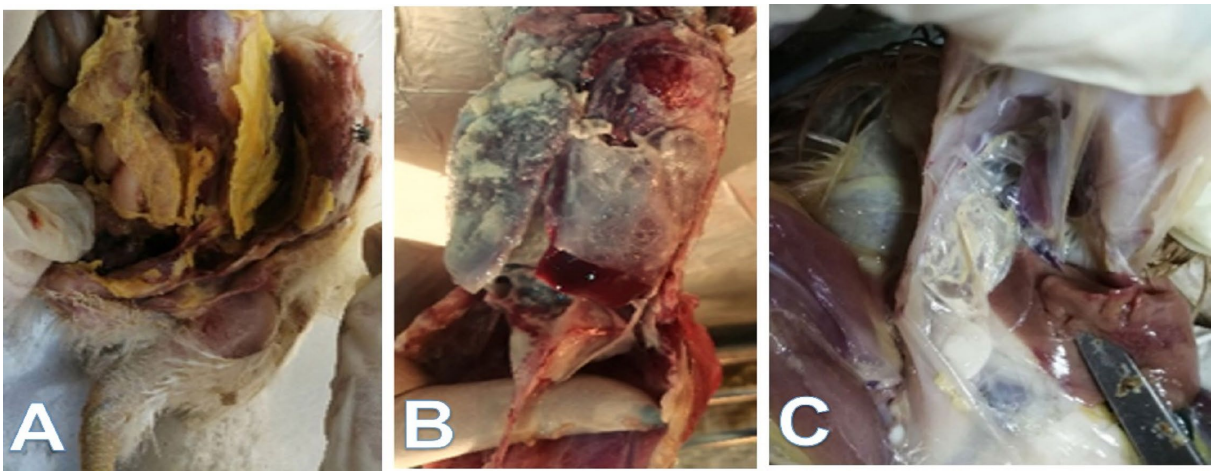

Fig. 1. Postmortem of freshly dead chickens showing A and B: Caseous and fibrinous pericarditis, perihepatitis and air sacculitis in broiler chickens; C: Foamy air sacculitis in layer chicken.

acid analysis and nucleotide analysis. To get rid of undetermined nucleotides, the original sequence was trimmed. The sequencing reaction often starts with sequences. one *mgc2* sequence was sent to the GenBank database, where the accession number was obtained OQ129762 (2656068 seq 1). utilizing the BLAST search algorithms and PSI-BLAST, respectively on the National Center for Biotechnology Information "NCBI" website, similarities between the nucleotide and amino acid sequences of the Egyptian MG strains and other strains published on GenBank was found. The BioEdit sequence alignment editor was used to produce the observed nucleotide sequences and the deduced amino acid of Egyptian MG strains and other strains reported on GenBank. Then (MegAlign) software was used to create the phylogenetic tree.

The single sequenced isolate in this study is grouped with the field strain from Egypt discovered by Eissa *et al*. (2011) and it differs from other field isolates from Egypt and the vaccinal F strain

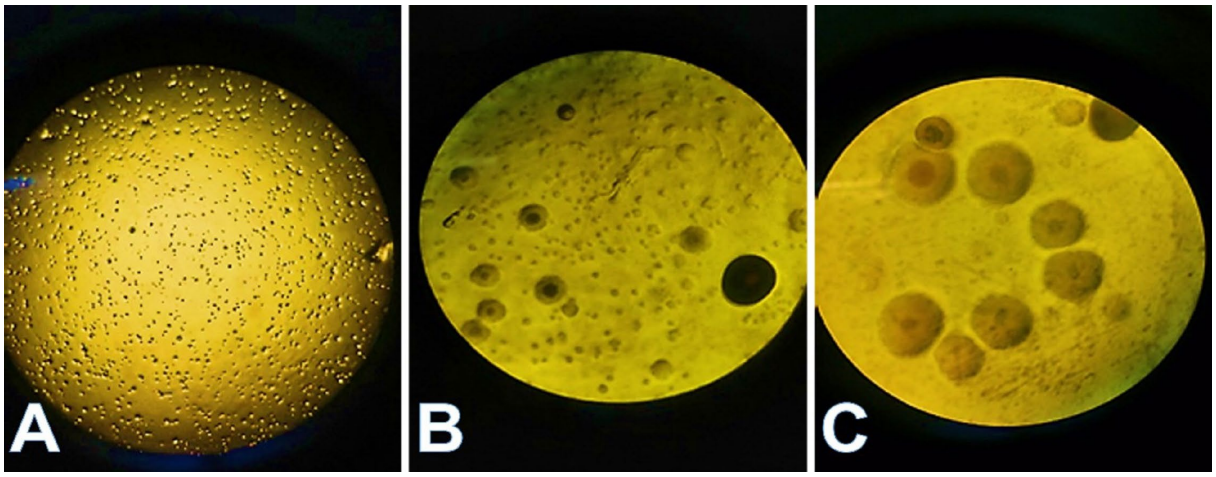

Fig. 2. Microscopical apperance; A: Fried egg apperance of MG at 10x ; B: Fried egg apperance at 25x of MG: ; C: Fried egg apperance at 40x of MG.

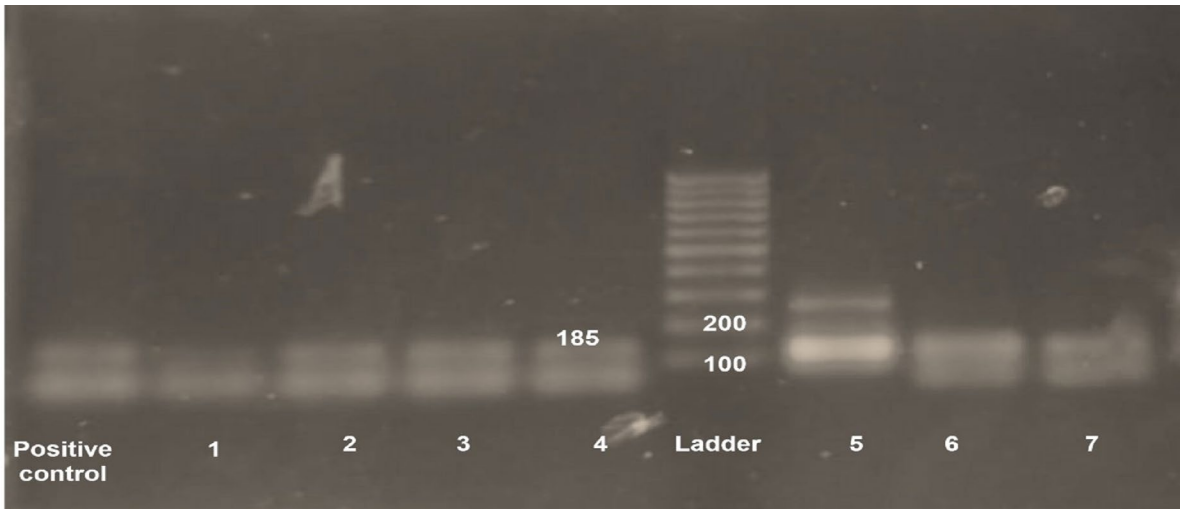

Fig. 3. Ultraviolet transilluminator photo showed 16s RNA, PCR results where lane from 1, 2, 3, 4, 5, 6 & 7 were positive at 185 bp.

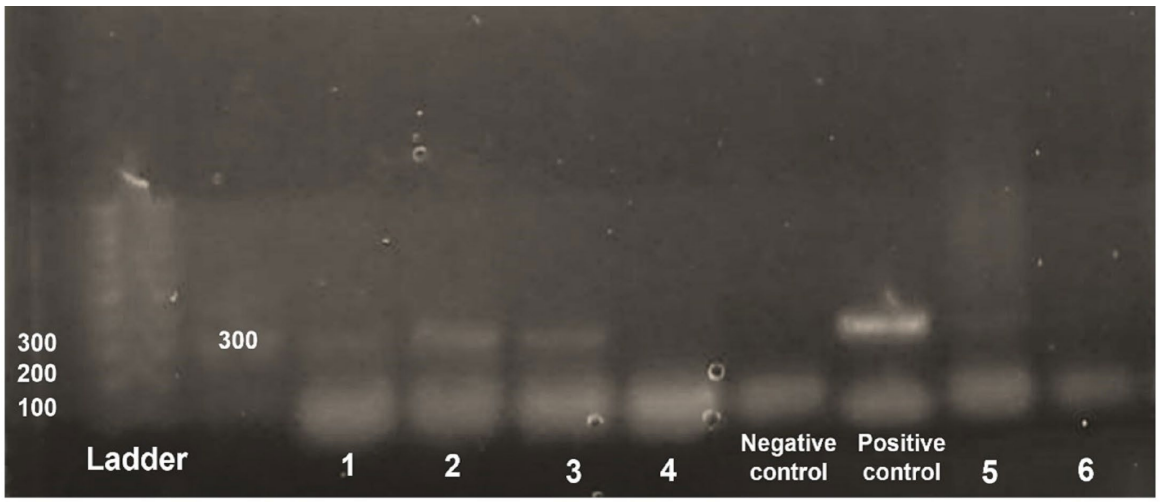

Fig. 4. Ultraviolet transilluminator photo showed *mgc2*, PCR results where lane number 1, 2, 3 & 5 were positive at 300 bp but other lane number 4 & 6 were negative.

with accession number (HQ591357.1) as in Figure 5.

DISCUSSION

Poultry farming is one of Egypt's most significant agricultural sectors, contributing significantly to the nation's supply of animal protein (eggs and meats) (Marouf *et al*., 2021; Abdelfatah *et al*., 2023). Worldwide, the chicken sector is seriously threatened by respiratory problems (Al-Shekaili *et al*., 2015; Setta *et al*., 2023). MG is a significant poultry disease that adversely affects egg hatchability and production as well as causes CRD (Swayne *et al*., 2013). Additionally, MG infections impact virus titers and the effectiveness of several poultry viral vaccinations (Fathy *et al*., 2017). Based on both traditional and modern methods, the current analysis demonstrates the prevalence of *Mycoplasma* in several Egyptian governorates and detection rate of MG from certain farms with flocks of broiler, and layer farms of various ages revealed that this organism is widely spread among the investigated Egyptian governorates.

According to our findings, the flocks of chickens under investigation displayed a range of respiratory symptoms, and PM examinations revealed varying degrees of air sacculitis, perihepatitis, pericarditis and serositis. Similar clinical signs and PM findings were reported by Emam *et al*. (2020).

MG infection is more common in the winter and autumn than in the summer and spring and these could be contributed to the widespread respiratory disease infections (viral and complicated bacterial pathogens) in the cold seasons as the low-temperature stress and elevated humidity increase the susceptibility to the respiratory tract to be infected with various pathogens (Setta *et al*., 2018).

The bio-typing characteristic of MG shows a ratio of 101/101(100%) (digitonin sensitive). Similar results were recorded by Marouf *et al*. (2021).

Since a serological test cannot identify a subclinical or early infection, the diagnosis of the causal agent in *Mycoplasma* is regarded as the gold standard (ley, 2003). Therefore, the identification of *Mycoplasma* in the current investigation was based on the use of culture and PCR techniques.

In this study the isolation rate during the period from March 2020 to January 2022 in different Egyptian governorates was 90.65% in broiler chicken and 66% in layer chickens. This isolation percentage was near to that recorded by Emam *et al*. (2020) and Marouf *et al*. (2021). However, Osman *et al*. (2009) and Muhammad *et al*. (2021) study showed that layer flocks are more common. These variation in isolation rate could be contributed to the difference in time, location, managemental procedures, treatments, vaccination programs applied during the samples collection and isolation procedures.

Traditional, conventional techniques for isolating and identifying MG are time-consuming, labor-intensive, less sensitive, and fail to identify *Mycoplasma* species from treated birds (Emam *et al*., 2020). The PCR approach, meanwhile, provides a rapid, sensitive, and precise way to identify MG from probable cases (Tomar *et al*., 2021).

On the base of molecular detection in this study, Figures 3 and 4 demonstrate that 101 colonies produced PCR-amplified fragments at 185 and 300 bp based on the 16 srRNA gene and the *mgc2* gene, respectively, with a ratio of 87.1% that largely agrees with those obtained by Emam *et al*. (2020); Marouf *et al*. (2021) and Mahmmoud *et al*. (2022) who reported that the prevalence of *Mycoplasma* was 87.5, 85, and 85.9%, respectively. The PCR offers several benefits, the development of contamination brought on by inadequate sample handling, however, limits it and causes false-positive results according to OIE (2004). Therefore, traditional cultural methods should be applied in parallel to PCR (Marouf *et al*., 2021).

This study's sequencing of one isolate's *mgc2* gene is grouped with the field strain isolated from Egyptian chickens reported by Eissa *et al*. (2011) with 100% identity who proven that the *mgc2* gene could discriminate between MG field type and vaccinal strains when F-strain was isolated from a commercial layer flock that had received the F-strain vaccination, this strain is closely related with neighbor strain which isolated from India 2018 by Rajkumar *et al*. (2019) with accession number (KP279741), who shared 100% identity with the foreign strains which isolated from THA 3CK08 (Thailand) and UHP1CK99 (Israel), this strain is closely related with other neighbor strain else which isolated from Iran 2019 by Norouzian *et al*. (2019) with accession number (MG428412), who demonstrated that two of the MG isolates under study contained a 22-amino-acid insertion that had not been seen in MG strains from other vaccines or the reference strain the observed diversity of common MG isolates from both backyard and commercial flocks when creating the MG management approach. Because of the detected gene mutations, more research is

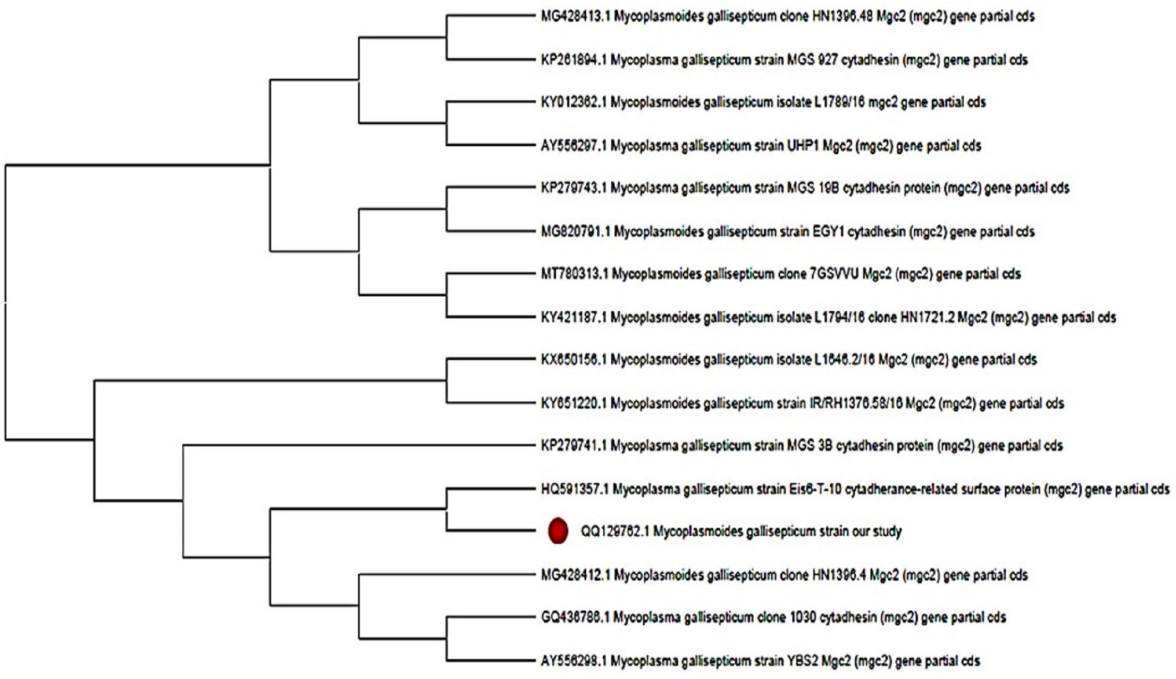

Fig. 5. Phylogenetic tree of *Mycoplasma gallisepticum* OQ129762 our study strain with other related strains.

required to comprehend changes in MG antigenicity and pathogenicity. So, efforts should be increased to lessen the detrimental effects of mycoplasmosis in the chicken sector by implementing biosafety controls and efficient vaccines in the breeding flocks to reduce the disease's vertical transmission (El-Naggar *et al*., 2022).

CONCLUSION

Mycoplasma gallisepticum is considered a widespread pathogen that causes respiratory disease in chickens among the several Egyptian governorates (Giza, Al-Qaliobia, Al- Dakahlia, Al-Faiyum and Al-Sharqia). *Mycoplasma gallisepticum* detection using PCR-based techniques is more accurate and rapid than classical culture-based techniques, they should be utilized in parallel with traditional culture methods. The *mgc2* gene could discriminate between MG field type and vaccinal F-strain. Periodical molecular monitoring is recommended to detect the variation in the circulated strains and further control and prevention techniques should be evaluated to overcome such problems.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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