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Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Fertile Eggs by ELISA and Real-Time PCR

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ABSTRACT

This study aimed to assess the occurrence of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) infections in fertile eggs using Enzyme-linked Immunosorbent Assay (ELISA) and Real-Time PCR. A total of 62 yolk samples were collected from August 2022 to April 2023. Of these, 31 samples (50%) were tested positive for antibodies via ELISA, while 13 samples (21%) were positive using Real-Time PCR. Specifically, 10 samples (16.12%) were positive for MG, and 3 samples (4.83%) were positive for MS, indicating a higher incidence of MG than MS in the hatcheries examined. The results suggest that maternal antibodies detected by ELISA may confer protection to chicks against MG and MS. This study confirms the vertical transmission of MG and MS from hens to their fertile eggs. The findings underscore the need for enhanced quality control measures and better management practices in hatcheries to mitigate the risk of Mycoplasma infections and improve overall poultry health.

Keywords: M. gallisepticum, M. synoviae, vertical transmission, ELISA, Real-Time PCR

INTRODUCTION

Avian mycoplasmosis was first identified in turkeys in 1926 and later in hens in 1936 (1). Among the causative agents, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are of greatest concern due to their substantial impact on the poultry industry (2). These pathogens are included in the list of diseases requiring notification by the International Organization for Animal Health (3). MG infection in chickens typically manifests as chronic respiratory disease, with common symptoms such as coughing, nasal discharge, sneezing, and labored breathing. In turkeys, MG is associated with infectious sinusitis, characterized by respiratory sounds, nasal secretions, and sinus swelling (3, 5, 6). MS causes infectious synovitis, a persistent and contagious disease ranging from mild to chronic, affecting the synovial linings of joints, tendons, and their sheaths in both chickens and turkeys (1). Furthermore, Feberwee and Landman (7) established a connection between MS infections and apical eggshell abnormalities in various countries.

These diseases are economically significant due to their efficient transmission mechanisms, including vertical transmission through eggs and rapid spread in hatcheries. The transmission rate is influenced by the bacterial strain and infection stage (8–10).

In Iraq, the diagnosis of Mycoplasma infections in hens has been performed since 1989, using culture methods (11–13) and later supplemented by polymerase chain reaction (PCR) (14–17). PCR is particularly valued for its sensitivity and rapidity in detecting *Mycoplasma* infections (18). Since traditional Mycoplasma isolation methods are time-intensive, PCR and serological assays have become crucial tools for the diagnosis and management of these infections (18–21). ELISA (enzyme-linked immunosorbent assay) has proven more reliable than the hemagglutination-inhibition (HI) assay for detecting MG infections, particularly in exhibition birds, as demonstrated by comparative serological studies (22–24).

Vertical transmission of MG from infected hens to their offspring has been well-documented (24). Previous studies have often employed embryonated eggs to investigate pathogen presence and antibody transmission. Egg yolk sampling for antibody detection using ELISA provides several advantages over blood sampling, including cost-effectiveness, convenience, and ease of collection (25–27). Studies by Hagan et al. (28) and others highlight the efficacy of egg yolk-based ELISA, offering high antibody concentrations and economical testing. This approach is widely utilized in diagnosing poultry diseases, including Mycoplasma infections and Newcastle Disease (29–32). In Iraq, research by Ali (33) and Ali et al. (34) has revealed significant prevalence rates of MG (10%), MS (55%), and mixed infections (35%) in broiler and layer chickens, primarily using PCR for diagnosis.

The *mgc2* gene has emerged as a pivotal target for MG detection due to its role in bacterial adhesion to host cells. PCR assays targeting mgc2 enhance diagnostic accuracy and provide insights into pathogen virulence, with significant implications for disease management (35). For MS, the intergenic spacer region (ISR) between the 16S and 23S rRNA genes is a valuable tool for strain typing, offering detailed genetic differentiation despite its complexity for routine diagnostics (36).

In Iraq, data on *Mycoplasma* detection in fertile eggs remain scarce, with most studies focusing on post-hatch infections (11–17). This study aims to fill this gap by determining the prevalence of MG and MS in fertile eggs from Iraqi hatcheries using ELISA and real-time PCR. By leveraging these diagnostic methods, the study seeks to provide critical data for improving hatchery practices, strengthening biosecurity, and enhancing poultry health and production outcome.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was granted according to the local committee of care and use of animals in research at the College of Veterinary Medicine, University of Baghdad (Approval number P.G/1005 dated May 20th, 2022).

Sample Collection and Processing

Between August 2022 and April 2023, 400 fertile eggs were collected from eight hatcheries in Baghdad. The eggs were transported to the laboratory under controlled biosecurity conditions and stored at 4–6°C to maintain their quality until processing.

Sanitization of the eggs was performed following a modified protocol by Damaziak et al. (37). This involved washing the eggs with water and detergent, followed by sterilization with 70% ethanol. The eggs were then cracked into sterile containers, and the yolks were separated and transferred into 1.5 mL microcentrifuge tubes containing 700 µL of sterile 1× PBS. The tubes were vigorously mixed to ensure uniformity and stored at -20°C for subsequent analysis.

To optimize sample processing, a pooling strategy was employed. Yolk samples from 6–7 eggs were combined into a single 2 mL microcentrifuge tube. Each pooled sample (200 µL) was centrifuged at low speed for 60 seconds, and the supernatant was carefully transferred to a new 2 mL tube. Ultracentrifugation was performed at 14,000 rpm for three hours (Fisher Scientific, USA). A pellet was formed at the bottom of the tube, and the supernatant was reduced to approximately 250 µL by removing excess liquid. The remaining liquid and pellet were resuspended to prepare for DNA extraction.

Real-time PCR

Genomic DNA was extracted from the yolk samples using the ReliaPrep™ Blood Genomic DNA Miniprep Kit (Promega), following the manufacturer's protocol.

Real-time PCR was employed to detect MG and MS. The PCR primers targeted the *mgc2* gene for MG and the 16S–23S intergenic spacer region (ISR) for MS, as specified in Table 1 (39). Each reaction was prepared in a total volume of 10 µL, consisting of 5 µL of 2× master mix, 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 0.5 µL of probe (10 µM), and nuclease-free water to adjust the final volume. A template DNA volume of 3 µL was added to each reaction to achieve a final concentration of approximately 3 ng/µL, ensuring it did not exceed 20 ng total. Positive and negative controls were included in each run to verify the accuracy of the assay and to monitor for contamination. The temperature profile for the PCR amplification was implemented as follows: first denaturation at 95°C for 5 min, then proceed with 40 cycles for secondary denaturation at 94°C for 30 sec, then, annealing at 60°C for 30 sec, and the extension temperature at 72°C for 30 sec.

Table 1. Real-time PCR primers and probes (39)

Gene	Primer/ Probe	Sequence (5'-3')
mgc2	MGFrt	TTGGGTTTAGGGATTGGGATT
	MGRrt	CCAAGGGATTCAACCATC
	MGPrt	Texas Red- TGATGATCCAAGAACGTGAAGAACACC
16S-23S ISR	MSFrt	CCTCCTTTCTTACGGAGTACA
	MSRrt	CTAAATACAATAGCCCAAGGCAA
	MSPrt	FAM*-ATTCTAAAAGCGGTTGTGTATCGCT

MGFrt: Forward primer for *Mycoplasma gallisepticum*; MGRrt: Reverse primer for *Mycoplasma gallisepticum*; MGPrt: Probe for *Mycoplasma gallisepticum*; MSFrt: Forward primer for *Mycoplasma synoviae*; MSRrt: Reverse primer for *Mycoplasma synoviae*; MSPrt: Probe for *Mycoplasma synoviae*. *FAM: 6-carboxyfluorescein

Enzyme-Linked Immunosorbent Assay (ELISA)

Egg yolks were transferred to collection tubes, vigorously mixed, and diluted at a 1:2 ratio with sterile 1× PBS. The samples were then refrigerated at 4–6°C. Conventional indirect ELISA kits (ID Screen® MG/MS Indirect, Innovative Diagnostics, France) were used to detect antibodies specific to MG and MS, following the manufacturer's protocol. Results were tabulated and interpreted as outlined in Table 2.

Table 2. The manufacturer's guidelines for ELISA

S/P Value Range	ELISA Antibody Titer	MG and MS Immune Status
S/P ≤ 0.3	Titer ≤ 992	Negative
S/P ≥ 0.3	Titer ≥ 992	Positive

S/P Value Range: specifies the range of the sample-to-positive (S/P) ratio used to interpret the ELISA results. ELISA Antibody Titer: indicates the antibody titer corresponding to each range of S/P values. MG and MS Immune Status: provides the interpretation of the immune status against *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) based on the S/P value and antibody titer

Analytical Analysis

Data analysis was conducted using SAS (Statistical Analysis System, version 9.1). The chi-square test was employed to evaluate significant differences in proportions, while the McNemar test was used to assess paired proportions. A P<0.05 was considered statistically significant. The diagnostic performance of real-time PCR (gold standard) was evaluated using sensitivity, specificity, and accuracy metrics, calculated as follows:

$$\text{Sensitivity (Sn \%)} = \frac{\text{TP}}{\text{TP} + \text{FN}} \times 100\%$$

$$\text{Specifity (Sp \%)} = \frac{\text{TN}}{\text{TN} + \text{FP}} \times 100\%$$

$$\text{Accuracy (\%)} = \frac{\text{TP} + \text{TN}}{N} \times 100\%$$

Where: TP = True Positives, FP = False Positives, TN = True Negatives, FN = False Negatives, N=TP+FP+TN+FN.

The agreement between ELISA and PCR was evaluated using the kappa statistic, a measure of the consistency between two diagnostic methods beyond chance. Kappa values are interpreted based on standard thresholds: values less than 0.0 indicate no agreement, while values between 0.0 and 0.20 represent slight agreement. Fair agreement is indicated by values between 0.21 and 0.40, moderate agreement by values between 0.41 and 0.60, and substantial agreement by values between 0.61 and 0.80. Values between 0.81 and 1.00 indicate almost perfect agreement. In this study, the calculated kappa value was 0.03, suggesting slight agreement between ELISA and PCR results. This minimal overlap reflects differences in the methodologies, with ELISA detecting antibodies indicative of exposure and PCR targeting pathogen-specific DNA to confirm active infection.

RESULTS

ELISA and PCR Detection of MG and MS

Based on ELISA and Real-Time PCR results, a total of 31 samples (50%) out of 62 tested positives with titers (1,850 - 8,821) Table 3. Meanwhile, only 13 samples (21%) out of 62 were positive by real-time PCR. Table 4 revealed significant differences (P<0.01) between the percentage rate of isolation of MS and MG by ELISA and Real-time PCR. The results from real-time PCR revealed that 10 samples (16.12%) out of the total 62 tested were positive for MG (Figure 1), while only 3 samples (4.83%) were positive for MS (Figure 2). The incidence of MG (16.12%) in eggs from the hatcheries in this study was higher than that of MS (4.83%) (Table 5).

Table 3. ELISA results for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) antibody detection using Screen® MG/MS kit in fertile eggs from different hatcheries in Baghdad province

S/P Value Range	ELISA Antibody Titer	MG and MS Immune Status
0.002 - 0.141	17 – 538	Negative (31/62)
0.548 - 4.455	1,850 - 8,821	Positive (31/62)

Table 4. Comparison of detection rates between ELISA and PCR for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS)

Detection Method	Positive for Either (MG/MS) (%)
PCR	13/62 (21.0%)
ELISA	31/62 (50.0%)
P-value	<0.01

Table 5. PCR Detection of MG and MS in Fertile Eggs

Pathogen	Positive Cases (%)
Mycoplasma gallisepticum	10/62 (16.12%)
Mycoplasma synoviae	3/62 (4.83%)
P-value	0.04

Comparative Analysis of ELISA and PCR Results

A paired analysis of ELISA and PCR results (Table 6) revealed limited agreement between the two methods, with a weighted kappa value of 0.03 (95% CI: -0.170 to 0.235). The sensitivity of PCR relative to ELISA was 53.84%, and its specificity was 51.02%, resulting in an overall accuracy of 51.61%. The higher detection rate of ELISA likely reflects past exposure or maternal antibody transfer, while PCR specifically detects active infections

Table 6. Agreement Between ELISA and PCR for MG/MS Detection

	Real-time PCR Results		
ELISA Result	**Negative (-)**	**Positive (+)**	**Total**
Negative (-)	25	6	31 (50%)
Positive (+)	24	7	31
Total	49 (79.0%)	13 (21.0%)	62
Parameter			**Value**
Weighted Kappa			0.032
95% Confidence Interval			-0.170-0.235
Sensitivity (%)			53.84
Specificity (%)			51.02
Accuracy (%)			51.61

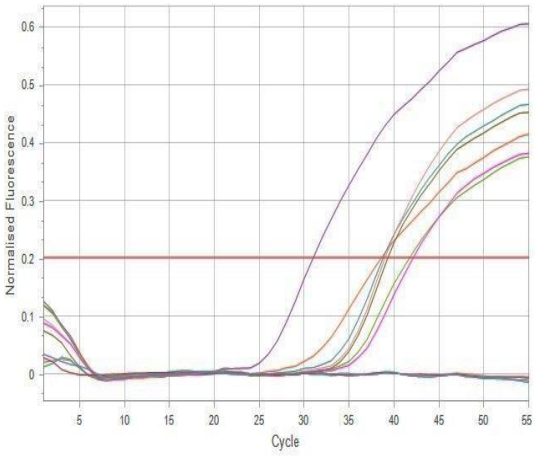

Figure 1. Plot of amplification of real time PCR for detection of *Mycoplasma gallisepticum* by amplification of the *mgc2* gene

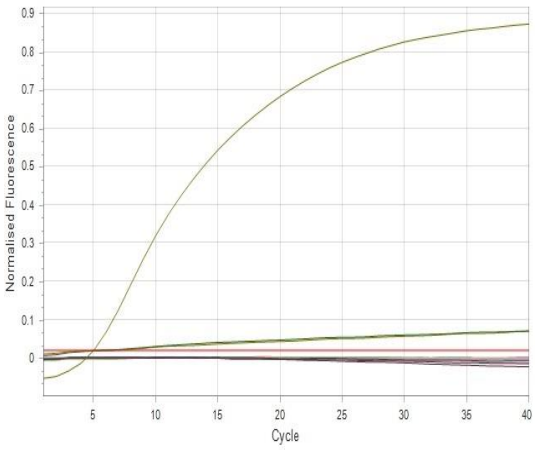

Figure 2. Plot of amplification of real time PCR for detection of Mycoplasma synoviae by amplification of 16S-23S ISR region gene

DISCUSSION

MG and MS are globally recognized as significant pathogens in poultry and are included in the list of causative agents of notifiable diseases (8). This study revealed a higher prevalence of MG and MS antibodies detected by ELISA (50%) compared to the detection rate of MG and MS DNA by PCR (21%). The ELISA results confirmed the presence of antibodies against MG and MS in egg samples, likely derived from maternal transfer, as hens exposed to or immunized against these infections pass antibodies to their eggs (5, 23). Previous studies have shown that these antibodies persist up to 18 days post-hatch, providing transient protection against field-strain MG in chicks (1, 41, 42). Maternal immunoglobulins are transferred to developing embryos via oviductal secretions and yolk deposition, highlighting the significance of maternal immunity in early chick development (43).

The higher positivity rate observed with ELISA compared to PCR can be attributed to the nature of the tests. ELISA detects antibodies indicative of exposure or immunity, while PCR identifies active infections by detecting the pathogen's DNA. This discrepancy reflects the timing and nature of pathogen transmission. Antibodies are transferred from hens to chicks through the yolk sac membrane during embryogenesis and circulate in the chick's bloodstream within a week of hatching (42, 44, 45). These findings align with previous comparative studies showing differences between ELISA and PCR in detecting MG and MS, focusing on the number of positive cases rather than antibody titers or DNA antigen levels (25, 26, 46).

ELISA remains a widely used, cost-effective serologic method for screening MG and MS infections. Commercial kits offer reliable detection of antibodies in various samples, including serum, respiratory secretions, and egg yolk. However, while tracheal swabs are traditionally used for MG isolation, egg yolks offer a practical alternative for monitoring Mycoplasma due to reduced contamination risks from natural microbiota (38, 41, 46).

This study demonstrated a 50% prevalence of MG/MS-positive cases by ELISA, lower than other studies such as one conducted in Vietnam, where 58.1% of MG cases were detected by PCR (48). Meanwhile, MS was detected at 9.3% in imported fertile eggs and 0.8% in locally produced fertile eggs. The vertical transmission of MG and MS was confirmed in this study, as evidenced by their detection in the yolks of fertile eggs.

The PCR-based detection of MG and MS in this study aligns with findings from Bagheri et al. (49), who identified MG in hatched embryos, healthy chicks, and breeding flocks in Malaysia. Notably, several samples tested negative for MG and MS in this study, suggesting the presence of other Mycoplasma species (50). The *mgc2* gene, encoding cytadhesin, a surface protein aiding MG adherence to host cells, was exclusively targeted for MG detection in this study. This gene has been established as a highly specific molecular marker for MG identification (53-55).

The real-time PCR results confirmed a higher incidence of MG (16.12%) compared to MS (4.83%), consistent with earlier findings. However, other studies have reported varying results. For instance, Galluzzo et al. (55) found a higher incidence of MS (23.25%) compared to MG (12.5%) in farm samples. Geographic variation was also noted in MG prevalence, with higher rates reported in France (68%) and Southern California (73%), while Central California reported only 3% (55). Comparatively, Iraq showed MG prevalence rates of 36.6% in broilers and layers (58), while MG prevalence was higher in Kuwait (58%), northern Pakistan (75%), and starlings (78.8%) (56, 57, 59). Variations in MG prevalence across studies may be attributed to differences in sample size, sample type, detection methods, flock age, breeder hen type, biosecurity practices, and geographic location (61, 62).

The findings highlight that PCR offers superior accuracy for detecting MG and MS compared to ELISA, particularly for active infections. The observed variability underscores the need for standardized sampling protocols and diagnostic techniques to improve consistency and comparability across studies.

This study demonstrates a higher prevalence of MG compared to MS in fertile eggs, indicating more efficient vertical transmission of MG. The presence of antibodies against MG and MS suggests maternal protection, although management and quality control of hatching practices require significant improvement. Enhanced biosecurity measures and rigorous quality control in hatcheries are essential to mitigate infection risks, ensure chick viability, and improve poultry production outcomes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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الكشف عن الميكوبلازما غاليسبتيكوم والميكوبلازما الزليلية في البيض المخصب بواسطة فحص الممتز المناعي المرتبط بالإنزيم و تفاعل البوليميراز المتسلسل

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الخلاصة

تهدف الدراسة إلى تقييم حدوث كل من عدوى المايكوبلازما غاليسبتيكوم (MG) والمايكوبلازما الزليلية (MS) في البيض المخصب في ضوء الفحوصات المصلية والجزيئية ، وتحديدا باستخدام فحص الممتز المناعي المرتبط بالإنزيم (ELISA) لتحديد الأجسام المضادة في صفار البيض المخصب واستخدام تفاعل البوليميراز المتسلسل لتشخيص MG-DNA و MS -DNA ، ،لهذ الغرض تم جمع ٦٢ صفار بيض مخصب خلال الفترة من أغسطس ٢٠٢٢ إلى أبريل ٢٠٢٣ من مفقسات مختلفة في بغداد. ما مجموعه ٣١ عينة إيجابية من أصل ٦٢ عينة بواسطة ELISA في حين أن ١٣ عينة إيجابية من أصل ٦٢ بواسطةPCR عشر عينات ٪16.12 PCR 10) كانت إيجابية ل MG و ٣ (٤,٨٣٪) عينات فقط إيجابية ل MS ، وخلال هذه النتيجة فإن حدوث اوانتشار بكتريا MG في البيض المخصب في المفرخات في هذه الدراسة هو أكثر من حدوث بكتريا MS. تحققت هذه الدراسة من عدوى الانتقال العمودي للميكوبلازما غاليسبتيكوم (MG) والميكوبلازما الزليلية (MS) في البيض المخصب لم يتم التعامل مع البيض المخصب ، الذي تم استخدامه في عملية الفقس في أفضل المواصفات وكانت تدابير مراقبة الجودة محدودة أو غير موجودة.

الكلمات المفاحية: المايكوبلازما غاليسبتيكوم ، المايكوبلازما الزليلية ، الانتقال العمودي، فحص الممتز المناعي المرتبط بالإنزيم ، تفاعل البوليميراز المتسلسل