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Detection of Mycoplasma gallisepticum in fertile eggs of chickens by culture and PCR

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ABSTRACT
This study aimed to assess the occurrence of Mycoplasma gallisepticum (MG) infections in fertile eggs using culture and molecular detection. 62 egg yolk samples were collected randomly from August 2022 to April 2023 and cultured on PPLO media for mycoplasma. By using the cultural approach, the rate of incidence of Mycoplasma was 51% (32/62)13\62 (20%) positive samples found in local fertile eggs while19\62 (30%) found in imported fertile eggs,19\62 (30%), we confirmed this result by (PCR) usingspecific primers targeting16SrRNA.The PCR 330bp product was sent to the MACROGEM(Korea)for sequencing, then submitted tothe Gene bank database with accession number: OR784572.1. Sequencing alignment showed that local (MG) isolates were highly identical with standard references at the gene bank, based on the nucleotide phylogenetic tree of the 16SrRNA gene we found that the Iraqi isolate was 100%similar to the USA, South Africa, United Kingdom Iraq: Baghdad Russia Australia isolates. Also, it was 99% identical to Japan, Iraq: Basrah, Urbana, Egypt, and India. This study confirms the vertical transmission of (MG) from hens to their fertile eggs Iraqi strains have high identical similarity, to the global strains.

Keywords: Mollicutes, 16S rRNA, sequencing, polymerase chain reaction, hatcher

INTRODUCTION

Mycoplasma gallisepticum is categorized as a bacterium belonging to the Mycoplasmataceae family and the Mollicutes class. The creatures under consideration are pleomorphic bacteria, characterized by the absence of cell walls and a high degree of surface protein diversity.(Feberwee, de Wit et al. 2022). Global recognition of M. gallisepticum as a highly pathogenic microbe has led to official listing and recommendation that it be reported to the World Organization for Animal Health (OIE) (Mycoplasmosis 2018). European Union Regulation (EU) 2016/429(Animal Health Law) and Regulation 2019/235 have set forth standards for monitoring poultry and hatching eggs as well as their trade within the EU and importation from non-EU countries. These laws aim to limit the transmission of MG infections.(Kursa, Tomczyk et al. 2024). A class of bacteria known as pathogenic mycoplasmas is accountable for significant financial losses in the world's chicken market. M. gallisepticum is a disease that significantly lowers the quality of carcasses and has significant economic implications. The mechanism by which M. gallisepticum spreads throughout flocks is linked to the high prevalence of the disease in chickens. Within a group of birds, mycoplasmas can spread both vertically and horizontally. Farm laborers, wild birds, and bird flocks can all spread these diseases (Dawood, Algharib et al. 2022). Birds can continue to function asvirus carriers, transferring it horizontally and vertically. Sadly, birds who have been infected with the virus will always carry the illness within them.(De Jong 2013). Infections with M. gallisepticum are among the costliest disease problems in chicken production because carrier eradication is required. Other factors that contribute to this include decreased feed intake, decreased egg production efficiency, and increased pharmaceutical costs. Serological procedures and culture methods are the two types of diagnostic approaches that can be applied in the process of diagnosing infections caused by M. gallisepticum. Numerous European countries have adopted preventive and control strategies focused on rigorous biosecurity, diagnostic monitoring, and the eradication of affected flocks.(Feberwee, de Vries et al. 2008, de Jong, Youala et al. 2021). Many

techniques that have been documented and applied have been used to identify M. gallisepticum in serum, egg yolk, and respiratory secretions.(Kachabi 2023). Even though M. gallisepticum is frequently found in chicken flocks, breeding flocks, and the eggs they lay, it can be periodically managed. Research done in Iraq by(Ali 2019, Ali and Yosif 2020)Showed that in broiler and layer-infected chickens with respiratory symptoms, the presence of MG (10%), MS (55%), and (35%) was positive for a mix of MG and MS. These findings were determined using a conventional PCR assay. To ensure the absence of M. gallisepticum in flocks, it is important to use replacement stock from a source that is free of this bacterium. As of right now, no research or data addressing the identification of Mycoplasma in chicken eggs is accessible in Iraq. Therefore, the primary goal of this investigation was to ascertain, via the use of culture and molecular features, the prevalence of M. gallisepticum in the yolk of fertile eggs in hatcheries.

MATERIALS AND METHODS

Samples Collection

400 fertile eggs were purchased and collected from different hatcheries in Baghdad from August 2022 to April 2023 after pooling 6-7 samples of yolk eggs to become 62 samples then we examined them for this investigation.

Samples Transport and Storage

On the day of collection, we carefully transported the eggs to the laboratory, ensuring their authenticity, integrity, and biosecurity. Subsequently, we stored the eggs in the refrigerator at a temperature ranging from 4 °C to 6 °C until they were processed.

Samples Processing

The eggs were sanitized by washing them with water and soap and then 70% alcohol to disinfect the outer layer, according to the modified process described by Damaziak et al.(Damaziak, Kieliszek et al. 2020). The eggs were cracked into individual jars, then divided and put into other containers. Within three days, the samples were assessed.

Culturing of yolk

According to Damaziaket al.(Damaziak, Kieliszek et al. 2020), the pooling approach involved combining 6-7 samples (yolk with vitelline membranes) into a single tube and mixing 200 of each sample.

Cultural of samples

0.2 ml of the sample was combined with 1-2 ml of mycoplasma broth (PPLO broth, Fisher, USA) that included growth supplements, and the mixture was thoroughly mixed. After that, the mixture was incubated for three days at 37 °C in an aerobic environment. A standard bacteriological loopful of the combination was streaked over (PPLO agar, Fisher, USA) and inverted after incubation. This streaking was carried out at 37 °C with elevated moisture and CO2 levels, either in a candle jar or an anaerobic jar. These circumstances persisted for six days. As recommended by (Sasipreeyajan, Halvorson et al. 1987, Abed 1988, Ali 2019, Basit, Mamun et al. 2021). Each plate was inspected for Mycoplasma proliferation every 3 days utilizing a microscope for dissection.

Subculture

By using a sterile loop to cut off an agar block with strong growth of Mycoplasma colonies, agar-to-broth transfer was carried out. The block was then re-incubated at 37 °C for 72hours. A loopful of the inoculum from the incubated broth culture was streaked out on PPLO agar before being kept in the broth at -20 °C to guarantee mycoplasma development in the broth. (Kareem 1991).

Isolation and purification of mycoplasma colonies.

Two techniques were applied to enhance the number of colonies on PPLO agar if the growth was sparse, each plate contains only 1 to 10 colonies. Each plate was filled with 1.5 ml of aseptic PPLO broth using the first procedure, and they were then incubated for an extra 48 to 72 hours at 37 °C. The second technique was inserting a tiny agar block with a single Mycoplasma colony into a screw-capped tube that contained sterile PPLO broth. A sterile loop was used to cut the agar block. Next, the tube was incubated for three to five days at 37°C. Ultimately, PPLO agar was subcultured onto a loop filled with the expanding PPLO broth.(Tiong, Liow et al. 1979, Quinn 2002, Ali 2019)

Detection of Mycoplasma gallisepticum by PCR

Genomic DNA was extracted from the positive colony using a DNA extraction kit according to the manufacturing protocol by ABIO pure Company.

Primers and PCR amplification

The PCR assay was conducted utilizing a specific primer a 330 base pair segment of a highly conserved section of the 16S rRNAgene in in (MG) designed by Kempf et al. (32) both primers were purchased from Macrogen Company. (Table 1)

Table 1: Primers used in the present study.

Gene		Sequence (5′-3′)	Product Size(bp)
16S rRNA	MG R	TAACTATCGCATGAGAATAAC	330
	MG F	GTTACTTATTCAAATGGTACAG	

Each PCR assay used a 25µl reaction volume containing 11.5µl of the 2 X PWO master mix (DNA Polymerase, reaction buffer with 4 mM MgCl2 and 0.4 mM each of PCR-grade dNTPs; Roche, Mannheim, Germany), 11.5µl of PCR grade water, 1µl of forward and reverse primers for 16Sr RNA (10µM each), and 1µl (30ng/µl) of genomic DNA template. The PCR amplification was conducted utilizing thermocycling conditions consisting of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The polymerase chain reaction (PCR) products were placed onto a 1.5% agarose gel saturated with ethidium bromide and subjected to electrophoresis for 1 hour at 100 V and 80 A. Visualization of the PCR products was achieved using a UV transilluminator.

DNA sequencing and phylogenetic analysis

The 330 bp PCR products were isolated from a gel of agarose utilizing a gel extraction kit of DNA from Qiagen, UK. A volume of 10µl of the purified PCR product was directed to the Bioneer Company in Korea for DNA sequencing utilizing both forward and reverse 16S rRNA primers on an AB DNA sequencing machine. The alignment of these sequences was performed and compared with other closely corresponding sequences using the NCBI BLAST (Basic Local Alignment Search Tool) from GenBank, and the ClustalW2 web software. The phylogenetic tree was constructed utilizing the Neighbor-Joining Method in the MEGA program as described.(Tamura, Dudley et al. 2007).

Statistical analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). The chi-square test was used to assess the significant difference between proportions.(Institute 2004).

RESULTS AND DISCUSSION

Throughout the current research (32) positive samples of yolk fertile eggs out of (62),13\62(20%) positive samples were found in local fertile eggs while from imported fertile eggs 19\62(30%) positive then The overall isolation rate of Mycoplasma spp. was (51.6%) by culture, the positive samples revealed the growth of mycoplasma colonies on PPLO agar such as typical fried egg colonies with or without regular edge and colonies with less center under stereomicroscope figure (1).The most dependable and gold-standard method for diagnosing M.gallisepticumis cultural isolation and identification, though it is time-consuming and difficult.It provides concrete proof of the existence of bacteria and is highly helpful for molecular strain typing(Al-Baqir, Hassanin et al. 2023). PPLO broth and agar medium with additives were determined to be acceptable for the growth of Mycoplasma, and they were used in the current study for the isolation of MG. This outcome is consistent with the research conducted by(Al-Delaify 1986, Abed 1988, Kareem 1991, Ali 2019).Various selective media, including Frey's medium, modified Hayflick medium, and Pleuropneumonia-like organism (PPLO) medium, have been used for isolation and assessed by various researchers(Heleili, Mamache et al. , Najeeb 2007, Ongor, Kalin et al. 2009, Rauf, Chaudhary et al. 2013).They found these medium are suitable for cultivation,The suspected colonies showed fried egg shape on solid media(García, Ikuta et al. 2005, S. Ehtisham-ul-Haque 2015) also appears another form of the colony with less center on solid media under a stereomicroscope (Al-Delaify 1986, Oie 2008, Al-Dabhawe, Kadhim et al. 2013). In the present study, there were 32 positive samples of yolk eggs out of 62, also the highest number of positive cases recorded in imported fertile eggs The overall isolation rate of positive Mycoplasma spp. colonies was 51.6%. This percentage is higher than the previous study by(Al-Delaify 1986, Kareem 1991, Ali 2019, Jafar and Noomi 2019)were isolated Mycoplasma at different localities from Baghdad with an incidence of (15.38%),(14.43%) and (23%) also higher than other prevalence of Mycoplasma in other countries (Singapore, Pakistan, and Egypt) by(Tiong, Liow et al.

1979, Abed 1988, Reda and El-Samie 2012, Rauf, Chaudhary et al. 2013)with prevalence percentages of (44.9%,14.4%, and 27.4%)respectively, the reasons for the high isolation rate of the present study because the trachea is routinely used to isolate. Previous studies have indicated that Mycoplasma has a propensity for multiplying in the site mentioned and has been commonly employed for isolation (Muhammad Mukhtar Muhammad Mukhtar 2012). Due to the presence of natural microflora, we have determined that using embryonated eggs as a sample is more effective than using tracheal swabs for detecting Mycoplasma spp., as it helps to avoid contamination issues. Consequently, egg samples can serve as a viable substitute for controlling MG (Hammouda, Pearce-Duvet et al. 2014). When reviewing earlier studies on embryonated eggs, researchers often focused on investigating egg yolks for M. gallisepticum.For instance, A comparative analysis was conducted between antibodies of egg yolk and antibodies present in serum samples utilizing the ELISA technique(Brown, Stoll et al. 1991, Hagan, Ashton et al. 2004, Hammouda, Pearce-Duvet et al. 2014, Daş, Hennies et al. 2017)M. gallisepticum is isolated from chicken reproductive samples from the air sacs, the surface of the ovaries (which are located near the abdominal air sacs), and the mucosa surface of the oviduct(Sasipreeyajan, Halvorson et al. 1987). It has been observed that M. gallisepticumorganisms can spread from the respiratory tract to the ovaries through the bloodstream, leading to pathological conditions. This indicates the tissue preference of Mycoplasma(Peebles, Parker et al. 2004). Figure (2) shows the PCR product analysis of the 16S rRNA gene in M. gallisepticum for the positive isolates (330bp It has been noted that M. gallisepticum organisms can disseminate from the respiratory tract to the ovaries via the bloodstream, resulting in pathological conditions. This indicates the tissue preference of Mycoplasma(Garcia, Jackwood et al. 1995, Nascimento, Nascimento et al. 1998, Feberwee, de Wit et al. 2022)used PCR with species-specific primers for easy identification and confirmation of MG in cultures. Our study used DNA extraction of some purificationpositive colony samples that have center which was confirmed by conventional PCR revealedthat the PCR product analysis of 16SrRNA gene in Mycoplasma gallisepticumpositive isolates at (330bp) PCR product 16SrRNA gene to confirmation of positive colony isolates of mycoplasma by culture by amplification of the 16S rRNA gene using M. galisepticum specific primers were yielded an amplification product of 330bp this result similar to different authors (n., Reza et al. 2005, SHAHRIARI, AFSHARIFAR et al. 2005, Gharaibe 2008, Muhammad Mukhtar Muhammad Mukhtar 2012, Hossam, Wagih et al. 2016, Kursa, Tomczyk et al. 2024)were used same gene. The excessive proliferation of nonpathogenic mycoplasma species that grow at a quicker rate, as well as the presence of other organisms or the absence of growth in subsequent cultures, can hinder the cultivation of MG from clinical samples in the laboratory(Mallinson and Rosenstein 1976, Garcia, Jackwood et al. 1995, Nascimento, Nascimento et al. 1998, Quinn 2002, Manual 2008, Reda and El-Samie 2012, Rasoulinezhad, Bozorgmehrifard et al. 2017, Waheed Khudiar Khareeb, Ismail Ibrahim et al. 2022).The phylogenetic analysis of 16S r RNA was conducted using an Iraqi field isolate deposited in the NCBI database under the accession number OR784572.1: M. gallisepticum: Iraq figure (3)

Table 2. Numbers and percentage of positive cases of Mycoplasma in fertile eggs of chickens

Source of fertile eggs	Samples	+ve cases (%)	Chi-square	P-value
Local fertile eggs	31	62\13(20%)	2.25	0.13 NS
Imported fertile eggs	31	62\19(30%)		
Total number of cases	62	62/32(51.6%)		

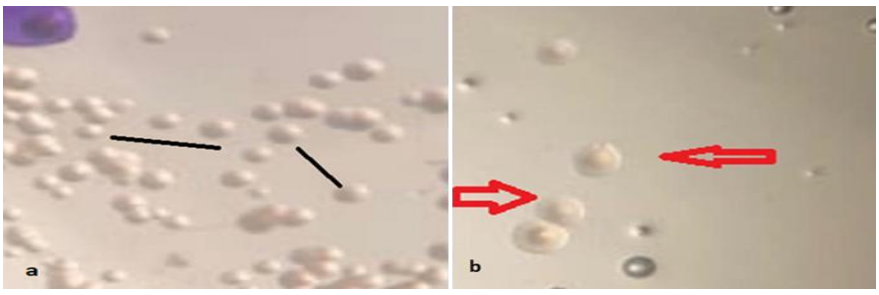

Figure 1. Culture characteristics of Mycoplasma colonies isolated from yolk eggs. (a) Fried-egg appearance (centerless) and (b) with center on PPLO agar under stereomicroscope (25X)

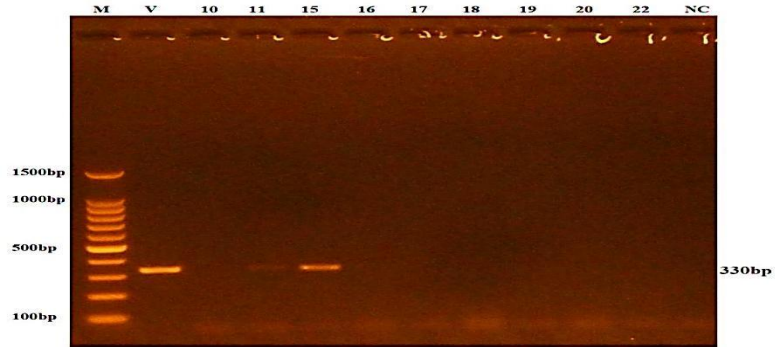

Figure 2. PCR amplicons of 16S rRNA gene in Mycoplasma gallisepticum isolated from eggs yolk Lane M: 100 bp DNA marker, Lane V: Positive control, Lane 15: Positive isolate showing a 330bp sized PCR product.

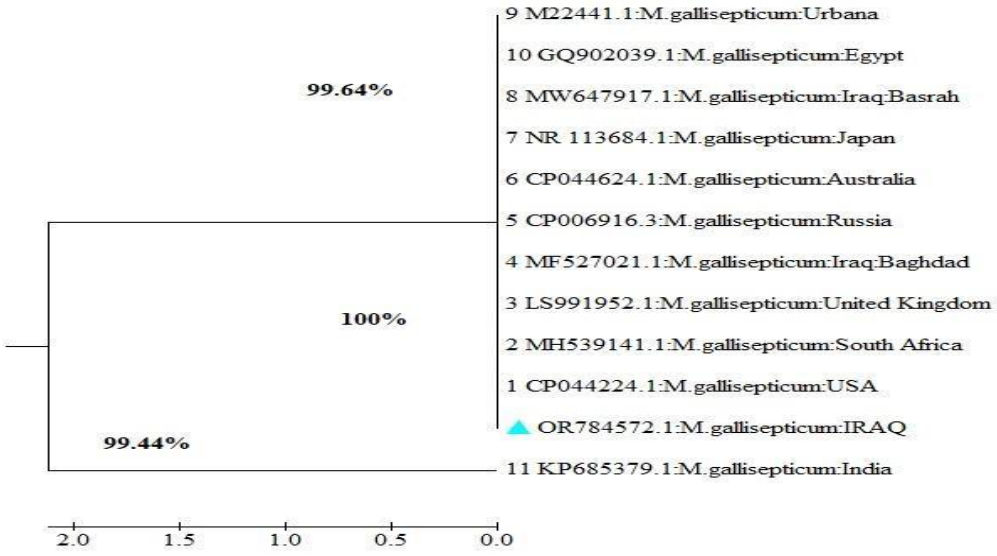

Figure 3. Phylogenetic tree of Mycoplasma gallisepticum isolated in this study based on a 330 bp fragment of 16S rRNA gene usingMega6 with NCBI.

According to the nucleotide phylogenetic tree of the 16S rRNA gene, we discovered that the Iraqi isolate had a 100% similarity to isolates from the USA, South Africa, and the United Kingdom. The countries mentioned include Iraq, Baghdad, Russia, and Australia Additionally, it is worth noting that Iraq's Basrah Urbana region, Egypt, and India have a striking similarity of 99% to Japan, as depicted in in Figure (3) and Table (2) Based on the examination of the phylogenetic tree of 16S rRNA incomplete nucleotide sequences of MG Iraqi strains/isolates and international strains, it can be inferred that Iraqi strains exhibit a high degree of similarity to global strains. The high ratio of sequence similarity suggests that there is a solid geographical spread of M. gallisepticum strains among neighboring nations. This is likely owing to insufficient biosecurity strategies, which implies an epidemiological and geographical relationship between these isolates.(Rasoulinezhad, Bozorgmehrifard et al. 2017). However, the overall management and quality control of the hatching process requires substantial improvements to ensure the health and viability of chicks. This emphasizes the need for enhanced handling procedures and rigorous quality control measures in hatcheries to prevent the spread of infections and improve egg and chick quality.Several samples appeared negative for MG and MS, which revealed the possibility of the existence of other Mycoplasma spp. (Ali 2019, Waheed Khudiar Khareeb, Ismail Ibrahim et al. 2022, Abtisam, Al-Awadi et al. 2023).

Table 2. Summarized the symmetrical sequence identity of local Mycoplasma gallisepticum. by NCBI-Blast based on 16S rRNA gene

	Source:Mycoplasma gallisepticum;16S ribosomal RNA gene				
	Accession	Country	Isolation Source	Date of registration	Compatibility
	ID: CP044224.1	USA	chicken	2019	100%
	ID: MH539141.1	South Africa	chicken	2018	100%
	ID: LS991952.1	United Kingdom	chicken	2018	100%
	ID: MF527021.1	Iraq: Baghdad	Gallus gallus	2017	100%
	ID: CP006916.3	Russia	chicken	2013	100%
	ID: CP044624.1	Australia	chicken	2019	100%
	ID: NR_113684.1	Japan	chicken	2011	99%
	ID: MW647917.1	Iraq: Basrah	chicken	2021	99%
	ID: M22441.1	Urbana	chicken	1993	99%
	ID: GQ902039.1	Egypt	chicken	2009	99%
	ID: KP685379.1	India	chicken	2015	99%

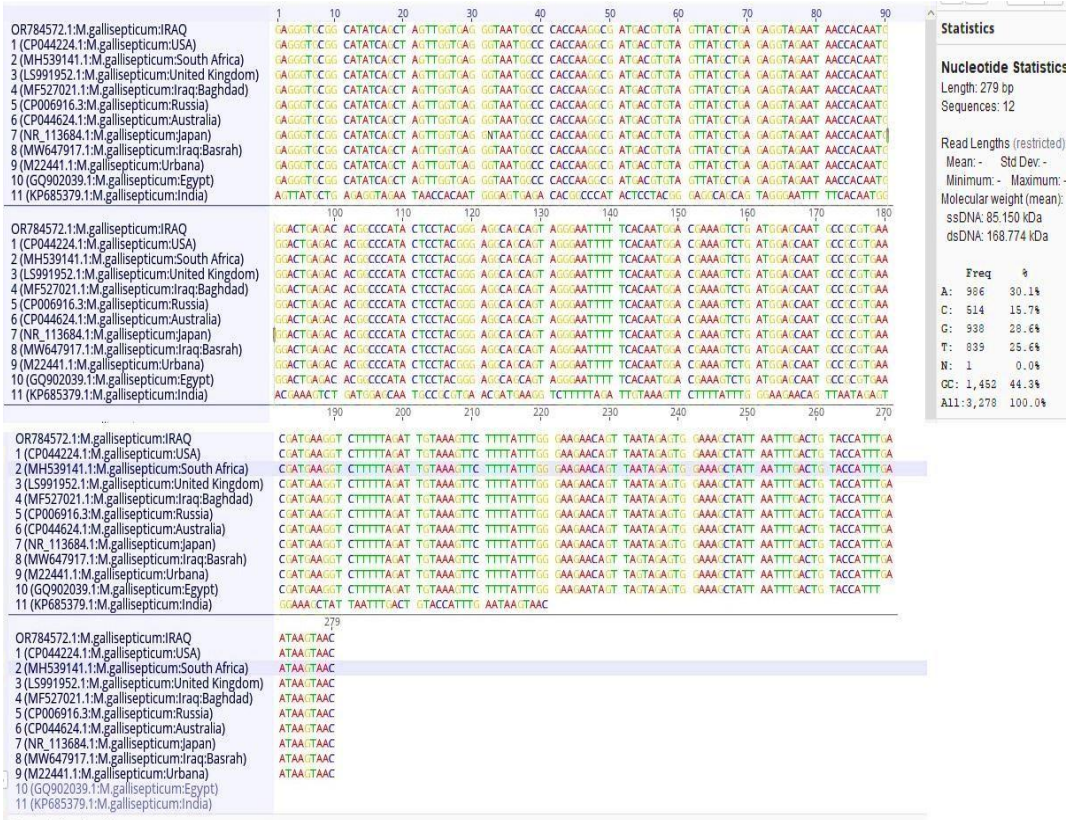

Figure 4. DNA sequence alignment of 16S rRNA. The alignment was done using ClustalW software. The sequence with OR784572 MG Iraq was isolated inthe present study. The other sequences were retrieved from the GenBank database.

Conflict Of Interest

The authors declare no conflicts of interest associated with this manuscript.

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REFERENCES

1. Abed, A. H., A.A.Al-Kaayyat,A.J.N. Al-Shammari (1988). "Effect of three Antibiotics on experimental infection of broiler with M. gallisepticum." The Iraqi Journal of Veterinary Medicine12(1): 285-298.
2. Abtisam, J., A. Al-Awadi and B. Ali (2023). "Comparative study of histopathological changes between nano-boron and boron in small intestine of layer chickens." Iraqi Journal of Agricultural Sciences 54(3): 724-729.
3. Al-Baqir, A., O. Hassanin, M. Al-Rasheed, M. S. Ahmed, M. H. Mohamed, M. S. El Sayed, M. Megahed, A. El-Demerdash, Y. Hashem and A. Eid (2023). "Mycoplasmosis in Poultry: An Evaluation of Diagnostic Schemes and Molecular Analysis of Egyptian Mycoplasma gallisepticum Strains."Pathogens12(9): 1131.
4. Al-Dabhawe, A., H. Kadhim and H. Samaka (2013). "Molecular detection of infectious bronchitis virus and it is relation with avian influenza virus (H9) and Mycoplasma gallisepticum from different geographical regions in Iraq." Iraqi Journal of Veterinary Sciences 27(2): 97-101.
5. Al-Delaify, S. (1986). "Study on Isolation and characterization of avian Mycoplasma In Iraq."
6. Ali, A. J. (2019). Identification and some Aspects Pathogenicity of Mycoplasma gallisepticum in broiler Chickens Doctor of Philosophy in Poultry Disease, University of Baghdad
7. Ali, B. and E. Yosif (2020). "Isolation and molecular characterization of Mycoplasma synoviae from infected chickens with respiratory signs." Iraqi Journal of Agricultural Sciences51(5).
8. Basit, M., M. Mamun, R. Md and M. Noor (2021). "Isolation and Molecular Detection of Mycoplasma gallisepticum in Commercial Layer Chickens in Sylhet." World's Veterinary Journal11: 614-620.
9. Brown, M., M. Stoll, A. Scasserra and G. Butcher (1991). "Detection of antibodies to Mycoplasma gallisepticum in egg yolk versus serum samples." Journal of Clinical Microbiology29(12): 2901-2903.
10. Damaziak, K., M. Kieliszek and M. Bucław (2020). "Characterization of structure and protein of vitelline membranes of precocial (ring-necked pheasant, gray partridge) and superaltricial (cockatiel parrot, domestic pigeon) birds." PLoS One 15(1): e0228310.
11. Daş, G., M. Hennies, B. Sohnrey, S. Rahimian, K. Wongrak, M. Stehr and M. Gauly (2017). "A comprehensive evaluation of an ELISA for the diagnosis of the two most common ascarids in chickens using plasma or egg yolks." Parasit Vectors10(1): 187.
12. Dawood, A., S. A. Algharib, G. Zhao, T. Zhu, M. Qi, K. Delai, Z. Hao, M. A. Marawan, I. Shirani and A. Guo (2022). "Mycoplasmas as Host Pantropic and Specific Pathogens: Clinical Implications, Gene Transfer, Virulence Factors, and Future Perspectives." Front Cell Infect Microbiol12: 855731.
13. De Jong, A., et al (2013). Mycoplasmosis. Diseases of Poultry: 875-941.
14. de Jong, A., M. Youala, U. Klein, F. El Garch, S. Simjee, H. Moyaert, M. Rose, A. V. Gautier-Bouchardon, S. Catania, K. Ganapathy, M. Gyuranecz, F. Möller Palau-Ribes, A. Pridmore and R. D. Ayling (2021). "Minimal inhibitory concentration of seven antimicrobials to Mycoplasma gallisepticum and Mycoplasma synoviae isolates from six European countries."Avian Pathol50(2): 161-173.
15. Feberwee, A., T. S. de Vries and W. J. Landman (2008). "Seroprevalence of Mycoplasma synoviae in Dutch commercial poultry farms." Avian Pathol37(6): 629-633.
16. Feberwee, A., S. de Wit and R. Dijkman (2022). "Clinical expression, epidemiology, and monitoring of Mycoplasma gallisepticum and Mycoplasma synoviae: an update." Avian Pathol51(1): 2-18.
17. Feberwee, A., S. de Wit and R. Dijkman (2022). "Clinical expression, epidemiology, and monitoring of Mycoplasma gallisepticum and Mycoplasma synoviae: an update." Avian pathology51(1): 2-18.
18. García, 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 Dis49(1): 125-132.
19. Garcia, M., M. Jackwood, S. Levisohn and S. Kleven (1995). "Detection of Mycoplasma gallisepticum, M. synoviae, and M. iowae by multi-species polymerase chain reaction and restriction fragment length polymorphism." Avian Diseases: 606-616.
20. Gharaibe, S., R.Dirgham and A.I.Roussan (2008). "The Use of Molecular Techniques in Isolation and Characterization of Mycoplasma gallisepticum from Commercial Chickens in Jordan." International Journal of Poultry Science7(1): 28-35.
21. Hagan, J. C., N. J. Ashton, J. M. Bradbury and K. L. Morgan (2004). "Evaluation of an egg yolk enzymelinked immunosorbent assay antibody test and its use to assess the prevalence of Mycoplasma synoviae in
UK laying hens." Avian Pathol33(1): 93-97.

22. Hammouda, A., J. Pearce-Duvet, T. Boulinier and S. Selmi (2014). "Egg sampling as a possible alternative to blood sampling when monitoring the exposure of yellow-legged gulls (Larus michahellis) to avian influenza viruses." Avian Pathology43(6): 547-551.
23. Heleili, N., B. Mamache and A. Chelihi "Incidence of avian mycoplasmosis in the region of Batna, Eastern Algeria."Hossam, M., A. Wagih, E. Mona, E. Mahmoud, E. Rehab and E.-D. T. Salah (2016). "The recovery and molecular diagnosis of Mycoplasma gallisepticum infection in commercial poultry flocks in Egypt." Ind J Sci Tech9: 29.
24. Institute, S. (2004). SAS/STAT 9.1: User's guide, SAS Institute.
25. Jafar, N. and B. Noomi (2019). "Detection of Mycoplasma gallisepticum and Mycoplasma synoviae by using of cultural and PCR technique." Iraqi Journal of Veterinary Sciences33(2): 469-473.
26. Kachabi, K. (2023). "Detection of Mycoplasma gallisepticum, Isolation, and Determination of Tylosin Susceptibility of Isolates from Commercial Chickens." Archives of Razi Institute78(4): 1247.
27. Kareem, E. A., A.J.N. Al-Shammari (1991). "Immunization of chicken against Mycoplasma gallisepticum." Iraqi J Microbiol 3: 117-122.
28. Kursa, O., G. Tomczyk, A. Sieczkowska, S. Kostka and A. Sawicka-Durkalec (2024). "Mycoplasma gallisepticum and Mycoplasma synoviae in Turkeys in Poland." Pathogens13(1).
29. Mallinson, E. and M. Rosenstein (1976). "Clinical, cultural, and serologic observations of avian mycoplasmosis in two chicken breeder flocks." Avian diseases: 211-215.
30. Manual, O. (2008). "Manual of diagnostic tests and vaccines for terrestrial animals." Chapter2(13): 304323.
31. Muhammad Mukhtar Muhammad Mukhtar, M. I. A., M. M. Awais, Nadeem Bhatti Nadeem Bhatti, Zahid Hussain Zahid Hussain (2012). "Elite Scientific Forum, Pathogenicity and effect of exogenous antibodies on the viability of Mycoplasma gallisepticum in chicken embryos." Journal of Life and Social Sciences10(1): 13-17.
32. Mycoplasmosis, W. O. A. (2018). "In OIE Terrestial Manual." WOAH: Paris, France3: 68-70.
33. n., G. G. B., S. A. Reza, A. Afsharifar and S. A. Pourbakhsh (2005). "Isolation And Detection Of Mycoplasma Gallisepticum By Polymerase Chain Reaction And Restriction Fragment Length Polymorphism." Iranian Journal of Veterinary Research6: 35-41.
34. Najeeb, A. H. a. M. I. (2007). "Comparison of Conventional Bacterial isolation, Rapid Slide Agglutination and Polymerase Chain Reaction for Detection of Mycoplasma gallisepticum in Breeder Flocks." Pakistan Journal of Life and Social Sciences5: 1-5.
35. Nascimento, E., M. Nascimento, M. Danelli, S. Machado, G. Lignon and P. de Polo (1998). Comparison of PCR kits for detection of Mycoplasma gallisepticum and M. synoviae (MS) in MS infected and noninfected chickens. Proceedings of... Western Poultry Disease Conference.
36. Oie, A. (2008). "Manual of diagnostic tests and vaccines for terrestrial animals." Office international des epizooties, Paris, France: 1092-1106.
37. Ongor, H., R. Kalin, M. Karahan, B. Çetinkaya and M. Akan (2009). "Detection of mycoplasma species in turkeys by culture and polymerase chain reaction." Revue scientifique et technique28(3): 1103.
38. Peebles, E., T. Parker, S. Branton, K. Willeford, M. Jones, P. Gerard, G. Pharr and W. Maslin (2004). "Effects of an S6 strain of Mycoplasma gallisepticum inoculation before beginning of lay on the leukocytic characteristics of commercial layers." Avian diseases48(1): 196-201.
39. Quinn, P. J., B.K.Markey ,M.E.Carter, W.J.Donnelly and F.C.Leonard (2002). . Mycoplasmas. In: Veterinary Microbiology and Microbial Disease, Black Well Publishing.
40. 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. Veterinary Research Forum,8(4):293–298
41. Rauf, M., Z. Chaudhary, M. Younus, A. Anjum, M. Ali, A. Ahmad and M. Khan (2013). "Identification of Mycoplasma gallisepticum by polymerase chain reaction and conventional diagnostics from white leghorn layer flocks."Reda, L. M. and L. El-Samie (2012). "Some studies on the diagnosis of Mycoplasma gallisepticum in chicken." Nature and Science10(12): 247-250.
42. S. Ehtisham-ul-Haque, S. U. R., M.I. Khan, M. Younus, M.M. Awais, A. Nasir (2015). "A simplified duplex real-time PCR incorporating TaqMan minor groove binder (MGB) probes and an exogenous internal positive control for the simultaneous detection of Mycoplasmagallisepticum and Mycoplasma synoviae cultures." Veterinarni Medicina60(5): 268–273.
43. Sasipreeyajan, J., D. Halvorson and J. Newman (1987). "Comparison of culturing Mycoplasma gallisepticum from fresh eggs and 18-day-old embryos." Avian diseases: 556-559.
44. SHAHRIARI, A. R., A. AFSHARIFAR and S. POURBAKHSH (2005). "Isolation and detection of Mycoplasma gallisepticum by polymerase chain reaction and restriction fragment length polymorphism."

45. Tamura, K., J. Dudley, M. Nei and S. Kumar (2007). "MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0." Molecular biology and evolution24(8): 1596-1599.
46. Tiong, S., T. Liow and R. Tan (1979). "Isolation and identification of avian mygoplasmas in Singapore." British Poultry Science20(1): 45-54.
47. Waheed Khudiar Khareeb, Z., Z. Ismail Ibrahim and F. Ali Abdullah (2022). "Pathological and Molecular detection of Mycoplasma ovipneumoneae in Sheep, Basrah Province, Iraq." Archives of Razi Institute77(6): 2073-2080.