Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

Evaluation of culturing and molecular assay for detection of *Mycoplasma gallisepticum* in chicken suffering from chronic respiratory disease

Dhiaa J. Hamzah[1,2*], Mdiha Ayed[3], Hayder A. Muhammed[4]

[1]*Department of Life Sciences, Faculty of Sciences- Sfax, University of Sfax, Tunisia*
[2]*Department of Pathology and Poultry Diseases, Faculty of Veterinary Medicine, University of Al_ Qadisiyah, Iraq*
[3]*Department of Animal Resources, Faculty of Agriculture Food and Rural Development, University of Sousse, Tunisia*
[4]*Department of Microbiology, Faculty of Veterinary Medicine, Karbala University, Iraq*

ARTICLE INFO

Original paper

Article history:
Received: December 09, 2021
Accepted: March 17, 2022
Published: April 30, 2022

Keywords:
Mycoplasma gallisepticum (MG), Molecular, ELISA, Chronic respiratory disease, Chicken

ABSTRACT

Mycoplasma gallisepticum (*M. gallisepticum*) is a bacterium that causes chronic respiratory disease (CRD) and infectious sinusitis (IS) in chickens and turkeys. Therefore, rapid and immediate diagnosis or regular detection of *Mycoplasma* may be of great help to early detection. 120 chicken layers, Within Karbala city, were carried out during their laying period on breeding flocks. The study proposed a promising method for isolation of *M. gallisepticum*, 120 tracheal swabs and blood samples from chickens in different dairy farms were used to analyze *M. gallisepticum* utility of PCR and culture. Compared with ELISA anti-IgG *M. gallisepticum*, the clinical specificity of PCR detection is 89.66%, the sensitivity is 86.36%, and the kappa coefficient is 0.817. Compared with the culture method, the specificity is 100%, the specificity is 45%, and the kappa coefficient is 0.543. Demonstrate the method's effectiveness and applicability as a standard method for mycoplasmas field diagnosis.

DOI: http://dx.doi.org/10.14715/cmb/2022.68.4.11

Introduction

One of the main obstacles to production in poultry farms, all over the world, is the spread of diseases that cause many complications and deaths. *Mycoplasma gallisepticum* (MG) is a highly infectious respiratory pathogen that can affect poultry; It is one of the contagious factors causing the economic loss of poultry breeders. Mycoplasma infection was mainly described in turkeys in 1926 and later in hens. In 1936 the *Mycoplasma gallisepticum* (MG) chicken sepsis infection is called chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys (1). It leads to disease characteristics in laying hens, broilers and poultry flocks through sneezing, tingling of the respiratory tract, coughing, nasal and eyes discharge the chicken (2).

Mycoplasmosis in poultry is very economical Poultry industry losses, especially chicken meat losses all over the world. In broilers, it will lower Weight gain, feed conversion efficiency decreases, increases increased mortality and conviction rates home. In breeders and pigs, the disease leads to a reduction in disease-laying eggs, an increase and decrease in fetal mortality Hatchability and quality of chicks (3,4). Chickens of all ages are susceptible to infection of Mycoplasma disease, but young birds are more susceptible to adults, in addition, The cost of medicines and vaccinations makes this disease one of the most common challenges and most expensive disease problems in the poultry industry (5). Mycoplasma can be detected in tissue parts of infected organs (such as the trachea and lung) and tracheal swabs (6).

Previous studies have described alternative laboratory markers for routine bacterial culture for the detection of *Mycoplasma gallisepticum* (7). These markers include serological testing for CRD infection to determine the presence of antibodies against *Mycoplasma gallisepticum* (8), and molecular testing for *Mycoplasma gallisepticum* (9).

Materials and methods

A total of 120 chicken layers (5- weeks-old chickens) were aggregated into 4 breeding flocks, Within Karbala city, 120 commercial layer chickens

*Corresponding author. E-mail: Dhiaa.hamzah@qu.edu.iq

were carried out during their laying period on breeding flocks, each of which had white leghorn chicken breed in the period between February 2020 and August 2021, and it involved four different regions of the city: Al-Hur, Al Husayniyah, Ain al-Tamr, and Al-Hindiyah.. All the flocks shared the same genetic background and were monitored in the same conditions. The flocks within the whole exhibited breathing difficulties and have not been treated with antibiotics. Within 6 hours after collection, all samples were sent to the laboratory on dry ice in the microbiology laboratory in the veterinary medicine college, Karbala University for culture, PCR, and ELISA assays.

Field samples processing

Swab samples were acquired by scraping the mucosal surface of the chicken trachea, partially cutting it off, and transporting it to the laboratory in microcentrifuge tubes containing 1 ml sterile physiological saline water. The diluted samples were used in the culture procedure, and the bacterium was confirmed using PCR analysis.

ELISA assay

With a 3 mL sterilized disposable plastic syringe, the blood sample was collected aseptically from each bird's wing veins and left to coagulate for 1 hour in the sterile tube. To obtain clear serum, tubes containing blood were kept in the refrigerator for 4-5 hours at 4°C. The serum (liquid portion) was decanted into a centrifuge tube and spun for 5 minutes at 2,500 rpm. The serum was then collected and stored in a sterile microcentrifuge tube at -20° C until it was processed for the serological investigation. ELISA kits designed specifically for MGs, these MG-specific ELISA kits (SunLong Biotech Co. LTD/China) were used to examine 120 blood samples from the same chicken laying at the same time, the commercial chicken layer under investigation had not previously been vaccinated. This kit's microplate is pre-coated with an anti-MG antibody, making it a solid-phase antibody. They are mixed with the antigen in the microplate wells. Each microplate well is then incubated with the antibody-antigen-enzyme labeled antibody complex, which is created in each well if the OD value is less than CUT OFF, which indicates that the sample is negative for chicken MG. Alternatively, if the OD value is greater than CUT OFF, the sample has been found to be positive for chicken MG.

Culture technique

A 100l aliquot of the vortexes 1ml sterile deionized water that included the tracheal swab was added to modified Frey's broth and cultivated at 37°C with 5% CO2 and high relative humidity for 24 hours (10). Every day, the color of Frey's broth changed, and the shift from pink to orange-yellow was regarded as a promising cultural sign. One week after incubation, cultured broths with unchanged colors were placed in new Frey's broth, followed by one additional passage if the color remained unchanged after one week. Positive broths were streaked on Mycoplasma modified Frey solid agar medium and incubated for at least 2 weeks at 37°C in a humidified environment with 5% CO2. Plates were examined for typical colonies under an inverted microscope. A digitonin test was used to identify Mycoplasma from Acholeplasma colonies once the colonies had grown sufficiently. Mg and Ms were identified using a growth inhibition assay with specific antisera (BioChek) as described (11).

PCR assay

A 100µl aliquot of the vortexed 1ml sterile physiological saline containing the tracheal swab was finely ground until they become emulsified broth, then centrifuged at 13,000 rpm for 5 minutes after being heated to 100°C for 10 minutes, and -20°C for 10 minutes. The supernatant was used directly extracted using a DNA extraction kit, then followed by used the forward primer 5- GCTTCCTTGCGGTTAGCAAC-3 and the reverse primer 5-GAGCTAATCTGTAAAGTTGGTC-3 to test the presence of MG. The PCR protocols: 94 °C for 5 minutes; 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds; and a final 5-minute extension at 72 °C (12).

Statistical analysis

Cohen’s kappa was used in the SPSS version (25.00) to analyze the agreement between culture and ELISA as well as and McNemar Test to analyze the agreement between different regions and different seasons of all samples.

Results and discussion

The cases included in this study consisted of 120 chicken layers, when evaluated according to the symptomatic signs, 55% of the IgG test was found to be positive, while 73.3% of the Mycoplasma culturing test was found to be positive in commercial layer farms.

Mycoplasma modified Frey solid agar medium: chicken layer with sample-based studies revealed that 87 (73.5%) of 120 pooled swab samples tested positive for MG, On agar plates, tracheal samples inoculated with Frey's broth and showing swirling growth with color change were subcultured in the Frey solid agar medium. These findings are based on the observation of mycoplasma colonies that have the typical fried egg appearance, those colonies were clarified under X10 dissecting microscope (Figure 1).

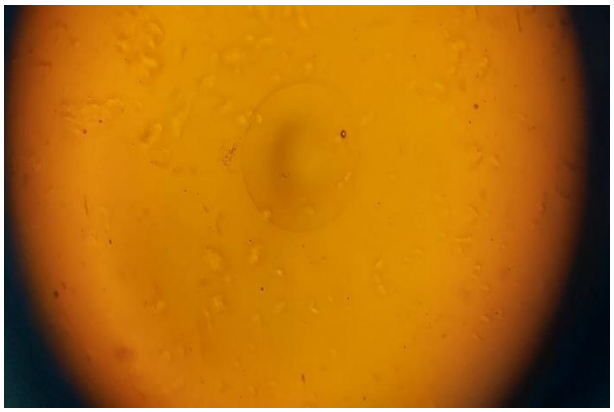

Figure 1. A typical single colony of *Mycoplasma gallisepticum* growth at 10X objective lens of dissecting microscope

Polymerase chain reaction (PCR) was used to identify suspected mycoplasmas (n=120) and verified isolates accounted for 50.9 % of the total (Table 1). The 185 base pair PCR amplicon product observed in an agarose gel electrophoresis revealed that the MG primers had successfully targeted the appropriate gene (Figure 2).

The seroprevalence of MG antibodies in commercial layer farms throughout the laying period of different Karbala city districts was established through the use of an ELISA test. 120 sera samples were collected and performed an ELISA test, with the results showing that 66 sera samples (55%) had positive results in Table 1, indicating that they had specific antibodies (IgG) against MG Figure (3).

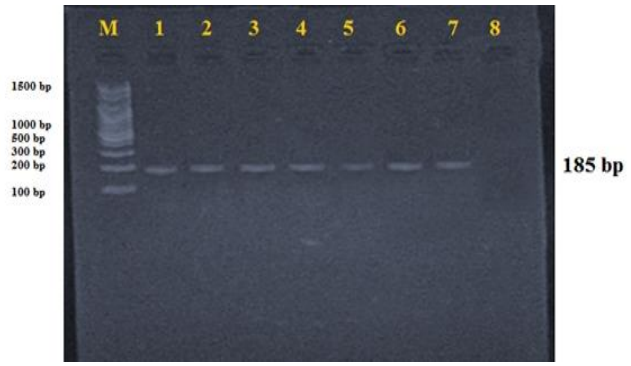

Figure 2. *Mycoplasma gallisepticum* was observed via DNA agarose gel electrophoresis analysis. Lane M represents a 1.5 kbp DNA ladder, Lane 1-7 represents a PCR result, and Lane 8 represents negative control.

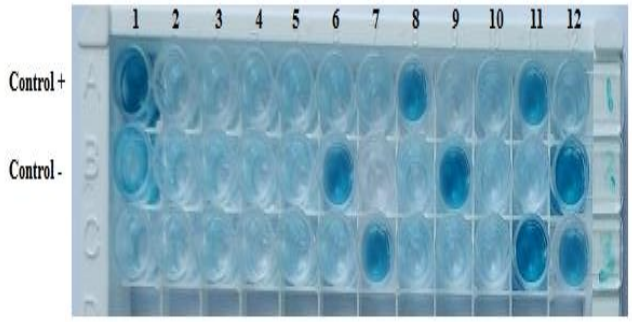

Figure 3. The presence and absence of antibodies to MG are determined by relating the value of unknown to the positive and negative control, blue color develops into a positive result if it is found more than the cutoff value.

On the farm, the prevalence of MG antibody and culture medium was 55% & 32.5%, respectively, Al-Hur Poultry flock was 30% and 10%, Al Husayniyah Poultry flock was 60% and 26.7%, Ain al-Tamr Poultry flock was 73.3% and 53.3%, and Al-Hindiyah Poultry flock was 56.7% and 40% by using ELISA and PCR, respectively Table (1).

We note from Table 1 that most cases of infection occurred in the Ain al-Tamr region in Karbala, but there are no significant differences between the bacteria isolated and areas by both tests.

Table 1. Prevalence of Mycoplasma gallisepticum among different regions in Karbala city

Karbala city (N)	ELISA test		PCR	
	+ (%)	- (%)	+ (%)	- (%)
Al-Hur (30)	11 (36.7)	19 (63.3)	14 (30)	16 (70)
Al Husayniyah (30)	16 (53.3)	14 (46.7)	12 (23.3)	18 (76.7)
Ain al-Tamr (30)	21 (70)	9 (30)	18 (43.3)	12 (56.7)
Al-Hindiyah (30)	17 (56.7)	13 (43.3)	15 (33.3)	15 (66.7)
Total (120)	66 (55)	54 (45)	59 (49.1)	81 (50.9)
Statistical analysis	X^2= 6.814, P>0.05		X^2= 2.849, P>0.05	

120 chicken are tested for disease mycoplasmas. 66 chicken have seropositive IgG in serum; 54 chicken are not diseased, it was noted that the prevalence reached 57 (48.48%) from the number 66 (55%) using the PCR test, As for the other remaining 9 (51.52%), the PCR could not detect mycoplasma, on the other hand, The PCR reaction showed that it is possible to detect mycoplasma samples 6 (11.11%) also from chickens that do not have an IgG antibody 54 (45%), it is also No positivity was detected with both kits in 48 (17.4%) of the 120 samples (Table 2). The sensitivity of PCR assay was 86.36% with confidence interval (75.69% to 93.57%) and specificity was 89.66% with confidence interval (78.83% to 96.11%). Cohen's Kappa coefficient test and the agreement between PCR and ELISA were assessed. From the total data, a Kappa statistic was recorded as 0.817 in the overall data, it appeared as almost perfect agreement.

Table 2. Specificity and sensitivity of the PCR assay kit when compared with ELISA Mycoplasma -IgG

		ELISA assay (Gold standard)		Total
		+	-	
PCR assay	+	57 True positive	2 False positive	59
	-	9 False negative	52 True negative	61
Total		66	54	120

In the Table (2), 66 chickens have seropositive IgG in serum; it was found that 39 chickens are cultured mollicutes colonies, and it was noted that the prevalence reached (59%) from the number 66 (55%) using Mycoplasma modified Frey solid agar medium, As for the other remaining 27 (41%), modified Frey solid agar medium could not isolate mycoplasma, on the other hand, The modified Frey solid agar medium showed that it is not possible to detect mycoplasma samples 0 (0%) also from chickens that do not have an IgG antibody 54 (45%), The sensitivity of Mycoplasma modified Frey solid agar medium was 59.09% with confidence interval (46.29% to 71.05%) and specificity was 100.00% with confidence interval (93.40% to 100.00%). Cohen's Kappa coefficient test and the agreement between Mycoplasma modified Frey solid agar medium and ELISA was assessed. From the total data, a Kappa statistic was recorded as 0.565 in the overall data, it appeared as moderate agreement.

Table 3. Specificity and sensitivity of the culturing technique when compared with ELISA Mycoplasma -IgG

		ELISA assay (Gold standard)		Total
		+	-	
Culturing	+	39 True positive	0 False positive	39
	-	27 False negative	54 True negative	61
Total		66	54	120

In hens, *Mycoplasma gallisepticum* (MG) is an infectious pathogen that causes a chronic respiratory disease known as avian Mycoplasmosis, e0gg complexes of various ages tested positive for MG, and in some regions of the world, this illness is ubiquitous in commercial chicken and turkey production, MG is responsible for several severe primaries and secondary bacterial poultry illnesses (13).

The traditional cultivation method has always been microbial as the excellent and technique for biological detection (14); so far, it is still used as mycoplasma isolated in the world. The culture method is based on the directed culture of mycoplasma cells, and the user can promote some supplements into the medium for mycoplasma growth, On the 1-2 week after the initial inoculation, the subculture of the liquid medium was inoculated on the agar plate (15). Mycoplasma will be the Formation of tiny colonies (diameter <100–400 μm) on the lipid medium, the morphology of the colony, is diverse, ranging from a typical fried egg shape to a more irregular shape (Figure 1). Mycoplasma colonies were tiny under the microscope, and the work of judging the colony requires laboratory staff to have relevant experience (16).

On Mycoplasma modified Frey solid medium, Acholeplasma species cannot be differentiated from Mycoplasma species, to discriminate between Mycoplasma and Acholeplasma, additional discriminatory tests are required (17). The sterol requirement of Mycoplasma and Acholeplasma is a significant distinction because Mycoplasma species cannot synthesis sterols or fatty acids, they must rely on exogenous sterol from the media (18). Exogenous cholesterol is not required for the proliferation of Aholeplasma species, digitonin can form a compound with sterol, preventing Mycoplasma from absorbing exogenous sterol but not Acholeplasma. As a result, digitonin inhibits the growth of Mycoplasma but not Acholeplasma (19).

Traditional culture and identification procedures can be alternative with the use of a particular DNA detection method, Although MG can be detected using DNA probes, it is now far more frequent to employ PCR to amplify specific segments of DNA in tested material (20). At least one commercially available MG DNA test employs PCR directly from swab material. A private laboratory creates a kit for detecting MG field strains as well as one for identifying vaccine strains. Several PCR-based "home" tests have also been published, including a multiplex PCR designed to detect four pathogenic avian mycoplasmas (21), but not validated with clinical samples. Kempf has mentioned a number of methods (22). Furthermore, Lauerman (1998) published a manual that includes a validated PCR test for MG, MS, and other avian mycoplasmas based on the unique sequences of the 16S RNA gene. In the United States, a PCR based on the MG mgc2 gene was developed (23), is becoming more widely used because the identification of Preliminary strain can be performed by sequencing the PCR product; unrelated strains may occasionally share the same sequence.

Nucleic Acid Amplification Techniques (NAT) is a method for detecting mycoplasma cells by amplifying and detecting specific mycoplasma genome conserved sequences. It is fast, convenient, and less affected by sample types. It has become a common method for laboratory detection of mycoplasma contamination (24). First established a method for detecting mycoplasma nucleic acid using traditional PCR technology in 1989, and the Mycoplasma NAT method has also undergone a continuous development process. At present, there are many types of Mycoplasma based on the NAT principle at home and abroad. In 1994, established (25) a mycoplasma multiplex PCR detection method using six upstream primers and three downstream primers by comparing the 16S sequences of the ribosomal RNA genes of 25 common mycoplasma.

They are some commercially available ELISA preparations for antibodies to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in the companies. Manufacturers' recommendations for cut-off levels to consider positive and doubtful reactions influence sensitivity to some extent. The sensitivity might occasionally be "muffled" to avoid the well-known cross-reactions between Mycoplasma gallisepticum and Mycoplasma synoviae. In an ELISA approach, a monoclonal antibody (MAb) that recognizes an epitope of a 56 kDa MG polypeptide is utilized (26). In this system, the ELISA plates are coated with a whole-cell MG antigen, and the tested sera are added as in the conventional indirect ELISA method, but the reaction is scored based on the amount of blockage that occurs when the Conjugated MAb. An advantage of this system is that be used for sera of any avian species without the need for adaptation.

As a result of the high specificity and sensitivity of serological tests in everyday practice, it is highly suggested that they be used to regulate flocks. it should remember that the ELISA test must establish a sensitivity and specificity test under the settings of their research laboratory certain authors should also point out that these assays have not been validated for use with sera from multi-day birds (27). The most commonly used tests are RSA, and ELISA, although others such as radioimmunoassay, and micro immunofluorescence tests have been described (28). The quantity of sera tested within a flock is determined by the level of detection and the required confidence levels, it is necessary to use serological test for mycoplasma detection because some of the farm-owned companies that use ELISA techniques to detect virus antibodies in large numbers of chicken sera may find this type of assay suitable for mycoplasmas (29).

Serology is commonly employed to detect MG-infected flocks because of the limits of mycoplasma culture or molecular detection, and because recurrent serological monitoring of flocks for a range of illnesses is standard in health screening programs. Therefore, the blood test is considered a gold standard test (30).

Mycoplasmas are diagnosed in 120 chickens. The prevalence of the PCR test reached 57 (48.48 %) of the 66 (55 %) chickens that have seropositive IgG in serum; 54 chickens are not ill. on the other hand, The PCR test did not find mycoplasma in the remaining 9 (51.52 %). The PCR reaction revealed that mycoplasma samples 6 (11.11 %) can be identified even in hens without an IgG antibody 54 (45 %) and that no positivity was detected with both kits in 48 (17.4%) of the 120 samples (Table 2).

The current study noticed an increase in the sensitivity and specificity of the PCR test when we consider the ELISA as a gold standard, and in this way, it is possible to adopt the two assays in diagnosing poultry mycoplasmas in the flock because the perfect agreement (0.817) between them is strong and compatible. This result disagreement with (KT, 2015) who found that serological tests could have a high rate of false-positives, and his results should not be based solely on one test system. In addition, he found that Nucleic Acid Amplification Techniques are a viable approach for confirming Mycoplasma-infected flocks in laboratories, as long as appropriate primers are chosen.

On the other hand, the current study showed an increase in the specificity value of 100% in relation to the use of mycoplasma culture due to the absence of a false positive value when compared with the ELISA assay. On the contrary, the study showed a decrease in the sensitivity value, which reached 45%b in relation to the mycoplasma culture (Table 3), This difference is due to the worker's experience during culturing, as well as time consumption, or the convalescence of poultry and the lack of mollicutes in their tracheal tubes because we are looking for anti-IgG and not anti-IgM.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

References

1. Yoder Jr H. Mycoplasam gallisepticum infection. Diseases of poultry. 1991; NII Article ID(10028214952):198-211.
2. Gondal M, Rabbani M, Muhammad K, Yaqub T, Babar M, Sheikh A et al. Characterization of *Mycoplasma gallisepticum* isolated from commercial poultry flocks. J. Anim. Plant Sci. 2015;25(1):108-113.
3. Osman K, Aly M, Amin Z and Hasan B. *Mycoplasma gallisepticum*: an emerging challenge to the poultry industry in Egypt. Revue scientifique et technique (International Office of Epizootics). 2009;28(3):1015-1023.
4. Rezaei-Nasab M, Komeili G and Fazeli-Nasab B. Gastroprotective effects of aqueous and hydroalcholic extract of *Scrophularia striata* on ethanol-induced gastric ulcers in rats. Der Pharmacia Lettre. 2017;9(5):84-93.
5. Nunoya T, Yagihashi T, Tajima M and Nagasawa Y. Occurrence of keratoconjunctivitis apparently caused by *Mycoplasma gallisepticum* in layer chickens. Veterinary Pathology. 1995;32(1):11-18.
6. Kahya Demirbilek S, Yilmaz O, Eyigor A, Temelli S and Carlı K. Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by Real-Time PCRs and *Mycoplasma gallisepticum*-antibody detection by an ELISA in chicken breeder flocks. Kafkas Universitesi Veteriner Fakultesi Dergisi. 2015;21(3):361-366.
7. Kang M, Gazdzinski P and Kleven S. Virulence of recent isolates of *Mycoplasma synoviae* in turkeys. Avian Diseases. 2002;46(1):102-110.
8. Parker T, Branton S, Jones M, Peebles E, Gerard P, Willeford K et al. Effects of an S6 strain of *Mycoplasma gallisepticum* challenge before beginning of lay on various egg characteristics in commercial layers. Avian Diseases. 2002;46(3):593-597.
9. Ganapathy K, Saleha A, Jaganathan M, Tan C, Chong C, Tang S et al. Survey of campylobacter, salmonella and mycoplasmas in house crows (*Corvus splendens*) in Malaysia. Veterinary Record. 2007;160(18):622-624.
10. Frey M. A medium for the isolation of avian mycoplasmas. American Journal of Veterinary Research. 1968;29:2163-2171 (NII Article ID: 10029282862).
11. Power J. Unilateral enlargement of the eye in chicks infected with a strain of *Mycoplasma gallisepticum*. Vet. Rec. 1976;99:102-103 (NII Article ID: 10029810428).
12. Gomes A, Costa L, Vilela D, Marques M, Carvalhaes A, Marin S et al. Detection of *Mycoplasma gallisepticum* in dead captive psittacines in Belo Horizonte, Brazil. Brazilian Journal of Poultry Science. 2010;12:75-78.
13. Kleven S. Control of avian mycoplasma infections in commercial poultry. Avian Diseases. 2008;52(3):367-374.
14. Almasian-Tehrani N, Alebouyeh M, Armin S, Soleimani N, Azimi L and Shaker-Darabad R. Overview of typing techniques as molecular epidemiology tools for bacterial characterization. Cellular, Molecular and Biomedical Reports. 2021;1(2):69-77.
15. Emam M, Hashem YM, El-Hariri M and El-Jakee J. Detection and antibiotic resistance of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* among chicken flocks in Egypt. Veterinary World. 2020;13(7):1410.
16. Rakovskaya IV, Ermolaeva SA, Levina GA, Barkhatova OI, Mukhachev AY, Andreevskaya SG et al. Microcolonies: a novel morphological form of pathogenic *Mycoplasma* spp. BioRxiv. 2019:535559.
17. Boonyayatra S, Fox LK, Gay JM, Sawant A and Besser TE. Discrimination between *Mycoplasma* and *Acholeplasma* species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and

conventional polymerase chain reaction. Journal of veterinary diagnostic investigation. 2012;24(1):7-13.
18. Rottem S. Membrane lipids of mycoplasmas. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1980;604(1):65-90.
19. Saito Y, Silvius JR and McElhaney RN. Membrane lipid biosynthesis in *Acholeplasma laidlawii* b: elongation of medium-and long-chain exogenous fatty acids in growing cells. Journal of bacteriology. 1978;133(1):66-74.
20. Feberwee A, de Wit S and Dijkman R. Clinical expression, epidemiology, and monitoring of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*: An update. Avian Pathology. 2022;51(1):2-18.
21. Wang H, Fadl A and Khan M. Multiplex PCR for avian pathogenic mycoplasmas. Molecular and cellular probes. 1997;11(3):211-216.
22. Kempf I, Gesbert F, Guittet M and Bennejean G. Mycoplasma gallisepticum Infection in Drug-treated Chickens: Comparison of Diagnosis Methods Including Polymerase Chain Reaction. Journal of Veterinary Medicine, Series B. 1994;41(1-10):597-602.
23. Garcia-Garibay MA. Crystalline molecular machines: Encoding supramolecular dynamics into molecular structure. Proceedings of the National Academy of Sciences. 2005;102(31):10771-10776.
24. Bernet C, Garret M, De Barbeyrac B, Bebear C and Bonnet J. Detection of Mycoplasma pneumoniae by using the polymerase chain reaction. Journal of Clinical Microbiology. 1989;27(11):2492-2496.
25. Wirth M, Berthold E, Grashoff M, Pfützner H, Schubert U and Hauser H. Detection of mycoplasma contaminations by the polymerase chain reaction. Cytotechnology. 1994;16(2):67-77.
26. Fiorentin L. Molecular characterization of selected *Mycoplasma* iowae surface antigens. Auburn University; 2000.
27. Alexander D. Newcastle disease (APMV-1). Poultry Disease, 5th edn,(Harcourt Publishers, London). 2001:259-268.
28. Karaca K and Lam K. A radioimmunoassay for the detection of antibodies to *Mycoplasma gallisepticum*. Avian Diseases. 1986;30(3):628-631.
29. Volokhov DV, Kong H, George J, Anderson C and Chizhikov VE. Biological enrichment of *Mycoplasma agents* by cocultivation with permissive cell cultures. Applied and environmental Microbiology. 2008;74(17):5383-5391.
30. Adair B, Burns K, McNulty M and Todd D. A study of ELISA systems incorporating pooled viral and *mycoplasma* antigen preparations for antibody screening of chicken sera. Avian Pathology. 1990;19(2):263-278.