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Comparative Study of Various Diagnostic Methods for Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Egyptian Chicken Flocks

Marwa Emam[1*], Yousreya Mohamed Hashem[2], Elshaimaa Ismael[3], Mahmoud El Hariri[4], and Jakeen El-Jakee[4]

[1]*VACSERA Holding Company for Biological Products and Vaccines. Cairo, Egypt*
[2]*Animal Health Research institute, Agriculture research center, Giza, Egypt*
[3]*Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt*
[4]*Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt*

*Corresponding author's E-mail: dr.memam2009@gmail.com

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ABSTRACT

The significance of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in the poultry industry underscores the critical importance of diagnosing avian mycoplasmosis within the field of veterinary medicine. The present study aimed to compare various diagnostic methods for detecting MG and MS in Egyptian Chicken Flocks. A total of 360 samples were collected from breeder, layer, and broiler chickens from four governorates in Egypt. Conventional isolation methods and polymerase chain reaction (PCR) were used for the direct detection of MG and MS, while serum plate agglutination test (SPA) and Enzyme-linked immunosorbent assay (ELISA) were used for detecting antibodies against MG and MS. The highest detection rate of MG was found in commercial layers, followed by breeders, and broilers. Regarding MS, the highest detection rate was found in breeders, followed by commercial layers, and broilers. By comparing the used diagnostic methods, MG and MS were determined by the SPA test (40% and 31.1% respectively), ELISA test (31.7% and 23.6%), PCR (16.7% and 11.7%), and by the conventional culture method (10.8% and 3.9%). It could be concluded that the serological methods and PCR gave better sensitivity than culture methods and can be used in the diagnosis of avian mycoplasmosis.

Keywords: Chicken, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Sensitivity

INTRODUCTION

Mycoplasma infection is a critical problem in veterinary medicine and in the poultry production industry (Qasem et al., 2015). Infections with *Mycoplasma gallisepticum* (MG) in poultry are linked with multiple disease conditions, including those affecting the respiratory and reproductive systems (Al-Baqir et al., 2023). *Mycoplasma gallisepticum* and *Mycoplasma synovia* (MS) are considered one of the most important avian *Mycoplasma* species in the commercial poultry industry (Felice et al., 2020). Chronic respiratory disorders are usually driven by MG infections that are characterized by sneezing and coughing besides nasal and ocular discharges (Raviv and Ley, 2013; Ghadimipour et al., 2018) while MS infections occur as subclinical upper respiratory tract infections and also air sac disease may occur. *Mycoplasma synoviae* may also cause an acute to chronic infectious disease in chickens called infectious synovitis (Ghadimipour et al., 2018). Enormous economic losses in the poultry industry can be caused by both *MG* and *MS* infections through weight gain loss and reduced meat quality in broilers, resulting in a severe drop in egg production in layers, and increasing embryo mortality in breeders (Messa Júnior et al., 2017). Isolation of the organism in a cell-free medium or direct detection of its DNA in infected tissues or swab samples and also serological diagnostic tests are widely used to detect the existence of MG or MS (OIE, 2008).

After an initial serological screening of suspected birds, mycoplasmosis diagnosis can be confirmed

by polymerase chain reaction (PCR) and culture (Muhammad et al., 2018). Identification of MG and MS through detecting their DNA (PCR) in field samples or by cultures (OIE, 2008). Identification of *Mycoplasma* isolates can be done through Mycoplasma media, biochemical, serological, or molecular tests, as well as serological analysis of host sera using Serum plate agglutination test (SPA), hemagglutination inhibition (HI) test, or ELISA (El-Ashram et al., 2021). It is preferable to use serological tests for flock screening rather than for testing individuals. The goal of the present study was to compare the occurrence of MG and MS in chicken flocks using serology, molecular, and culture methods.

MATERIALS AND METHODS

Ethical approval

The samples were collected from birds according to ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Veterinary Medicine and Cairo University.

Sampling procedure

The samples collected from commercial layer, broiler breeder, and broiler farms from Elgarbeya, Elfayoum, Eldakahliya, and Giza governorates with clinical signs suggestive of MG or MS infections were investigated from February 2019 to the end of December 2019. Tracheal swabs (n = 360) were collected for isolation by culture and PCR detection. Additionally, blood samples (n = 360) were collected (2 ml) from the same examined chickens in an EDTA tube to record antibodies against MG and MS using serum plate agglutination (SPA) and enzyme-linked immunosorbent assay (ELISA).

Culture detection

The collected samples were cultivated into Pleuropneumonia like organism (PPLO) broth and agar (USA) media supplemented with *Mycoplasma* Enrichment Supplement FD075 at 37^0C in a moist 10% CO_2 for 3-5 days (Kleven, 2003). Traditional identification methods, such as digitonin sensitivity (Freundt, 1983), glucose fermentation (Ernø and Stipkovits, 1973), arginine hydrolysis (Fenske and Kenny, 1976), and film and spot formation test (Krieg and Holt, 1984) were performed.

Serological tests detection

Blood samples (2 ml) were collected aseptically from the wing vein using sterile disposable syringes, and left to clot then sera were separated by centrifugation and stored at 4^oC till used. The SPA test was performed by mixing 30 ul of serum with an equal volume of standard crystal violet *MG* antigen and MS antigen (Intervet, MSD animal health, USA) as well and then left for 2 minutes at room temperature (Heleili et al., 2012). Positive sera samples were inactivated at 56^0C for 30 minutes and serial dilutions were retested to ensure positivity in the SPA test (OIE, 2008).

Recombinant protein-based indirect ELISA was used to detect antibodies against MG and MS based on indirect ELISA. It was used to detect anti-MG antibodies in chicken sera (ID Screen® *MG* Indirect, IDvet) commercial test kit (France) and anti-MS antibodies in chicken sera (ID Screen® *Mycoplasma synoviae* Indirect, ID vet) commercial test kit (France). The procedures were followed according to manufacturer instructions.

PCR detection

DNA was extracted from tracheal swab samples suspended in 1 ml of PCR-grade Phosphate buffer saline (PBS) in a 1.5 ml snap-cap Eppendorf tube. The suspension was centrifuged for 30 minutes at 14,000 g at 4°C. Using a Pasteur pipette, the supernatant was carefully extracted and the pellet was then suspended in 25 µl PCR-grade water. The tube and the contents were boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 g for 5 minutes. The supernatant contained the DNA. *Mycoplasma gallisepticum* and MS were detected using *16S* rRNA primers (OIE, 2008, Table 1). Each PCR tube was filled with a 45µl volume of the reaction mixture followed by the addition of 5 µl of DNA sample. The tubes were put in thermal cycles and ran through the following cycles, 40 cycles, 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension), 72°C for 5 minutes and soak at 4°C. Conventional 2% agarose gel electrophoresis was used to detect the Electrophoresis PCR products were detected by. Gels were observed using an ultraviolet transilluminator and photographed (Sambrook et al., 1989).

Statistical analysis

The results were analyzed using PASW Statistics, Version 18.0 software (SPSS Inc., Chicago, IL, USA). Data was displayed in tables as descriptive statistics (frequencies). Chi-square (χ^2) test for independence and Fisher's Exact test were used to examine the relation between the diagnostic method and the rate of positive results detected, as well as the relation between the type of poultry production and region and the detection rate of *Mycoplasma* spp. infection. A *p*-value < 0.05 was regarded as statistically significant.

Table 1. Primers used for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* detection by PCR

Microorganism	Gene	Primer 5'- 3'	Amplicon size	Reference
Mycoplasma gallisepticum	*16S*rRNA	F-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C R-GCT-TCC-TTG-CGG-TTA-GCA-AC	185 bp	OIE (2008)
Mycoplasma synoviae	*16S*rRNA	F-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A R-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-	207 bp	

RESULTS

The present study indicated that the highest detection *MG* rate was identified in commercial layers aged 15-40 weeks, followed by breeders aged 50-70 weeks, and then broilers aged 30-39 days (Table 2). For MS, the highest detection rate was identified in breeders followed by commercial layers and broilers (Table 2). By comparing different methods for diagnosis of *Mycoplasma* infection (Tables 2 and 3 and Figure 1), the highest detection rates of *MG* and *MS* were recorded by serological tests including the SPA test (40% and 31.1%, respectively) and ELISA test (31.7% and 23.6%). These were followed by PCR (16.7% and 11.7%) and then by conventional culture methods (10.8% and 3.9%). The occurrence rates of *MG* and *MS* were higher in Eldakahliya than in Elgarbeya governorate among layers and breeders, while no infection was recorded among the Giza layer farm by the different methods of diagnosis. The lowest detection rate was recorded in the Elfayoum broiler farm.

Table 2. Comparative techniques for detection of *Mycoplasma gallisepticum* among the examined chickens

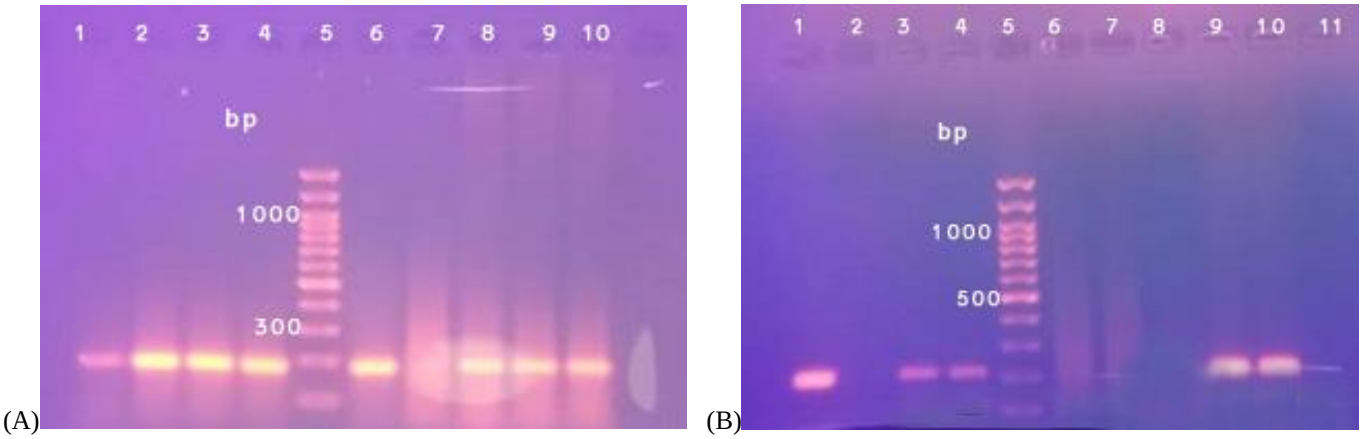
Parameters	Number of examined samples	Diagnostic method				*P*-value
		Culture No (%)	PCR No (%)	SPA No (%)	ELISA No (%)	
Type of poultry						
Broiler (30-39 days)	40	2 (5.0%)	4 (10.0%)	8 (20.0%)	7 (17.5%)	0.173
Layer (15-40 weeks)	170	21 (12.4%)	31 (18.2%)	74 (43.5%)	60 (35.3%)	< 0.0001*
Breeder (50-70 weeks)	150	16 (10.7%)	25 (16.7%)	62 (41.3%)	47 (31.3%)	< 0.0001*
	***P*-value**	0.403	0.454	0.022*	0.093	
Governorates						
Eldakahliya	200	27 (13.5%)	40 (20.0%)	95 (47.5%)	75 (37.5%)	< 0.0001*
Elgarbeya	100	10 (10.0%)	16 (16.0%)	41 (41.0%)	32 (32.0%)	< 0.0001*
Elfayoum	40	2 (5.0%)	4 (10.0%)	8 (20.0%)	7 (17.5%)	0.173
Giza	20	0	0	0	0	-
	***P*-value**	0.263	0.278	0.005*	0.047*	
Total	360	39 (10.8%)	60 (16.7%)	144 (40.0%)	114 (31.7%)	< 0.0001*

No: Number of positive results; *Indicate significance at $p < 0.05$. PCR: Polymerase Chain reaction, SPA: Serum Plate agglutination), ELISA: Enzyme Linked Immune Sorbent Assay

Table 3. Comparative techniques for detection of *Mycoplasma synoviae* among the examined chickens

Parameters	Number of examined samples	Diagnostic method				*P*-value
		Culture No (%)	PCR No (%)	SPA No (%)	ELISA No (%)	
Type of poultry						
Broiler (30-39 days)	40	1 (2.5%)	3 (7.5%)	6 (15.0%)	4 (10.0%)	0.277
Layer (15-40 weeks)	170	5 (3.0%)	17 (10.0%)	49 (28.8%)	38 (22.4%)	< 0.0001*
Breeder (50-70 weeks)	150	8 (5.3%)	22 (14.7%)	57 (38.0%)	43 (28.7%)	< 0.0001*
	***P*-value**	0.484	0.295	0.014*	0.041*	
Governorates						
Eldakahliya	200	10 (5.0%)	28 (14.0%)	77 (38.5%)	61 (30.5%)	< 0.0001*
Elgarbeya	100	3 (3.0%)	11 (11.0%)	29 (29.0%)	20 (20.0%)	< 0.0001*
Elfayoum	40	1 (2.5%)	3 (7.5%)	6 (15.0%)	4 (10.0%)	0.277
Giza	20	0	0	0	0	-
	***P*-value**	0.724	0.463	0.009*	0.009*	
Total	360	14 (3.9%)	42 (11.7%)	112 (31.1%)	85 (23.6%)	< 0.0001*

*Indicate significance at p <0.05. PCR: Polymerase Chain reaction, SPA: Serum Plate agglutination), ELISA: Enzyme-Linked Immune Sorbent Assay



(A) (B)

Figure 1. Agarose gel electrophoresis of amplified PCR products of *16SrRNA* gene among *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. **A:** Agarose gel electrophoresis of amplified PCR products of *16SrRNA* gene among *Mycoplasma gallisepticum* at 185 bp. Lane 5: 100 bp DNA marker (Thermoscientific), Lane 6: Positive control, Lane 7: Negative control, Lanes 1-4 and 8-10: *Mycoplasma gallisepticum* positive isolates. **B:** Agarose gel electrophoresis of amplified PCR products of *16SrRNA* gene among *Mycoplasma synoviae* isolates at 207 bp. Lane 5: 100 bp DNA marker (Thermoscientific), Lane 1: Positive control, Lane 2: Negative control, lanes 3, 4, 9, and 10: *Mycoplasma synoviae* positive isolates, Lanes 6-8: *Mycoplasma synoviae* negative isolates

DISCUSSION

The poultry industry plays a vital role for mankind through food supply (Fathy et al., 2017). Mycoplasma infection is considered a complicated and multifactorial disease causing economic problems to the welfare of poultry corporations in many parts of the world (Ibrahim et al., 2021). Highly significant avian *Mycoplasma* species in the poultry industry are *MG* and *MS* resulting in huge economic losses (Felice et al., 2020). *Mycoplasma gallisepticum* is a serious poultry pathogen causing enormous economic losses in the poultry industry as it causes a reduction in egg production, hatchability, and downgrading of carcasses (Ibrahim et al., 2018). Emam et al. (2020) revealed that the prevalence of *MG* among the examined birds was 9.85%, while *MS* prevalence was 1.6%. The present results indicated that the highest detection rate of isolation by culture of *MG* was obtained in commercial layers, followed by breeders, due to the long life span while the lowest detection rate is identified

in broilers. Abbas et al. (2018) reported that the lowest prevalence observed in broilers is due to the short life cycle before marketing leading to a minimum period of exposure. This finding is in correspondence with Osman et al. (2009) who indicated that the most affected birds originate from the layer flocks as the highest prevalence of *MG* isolation (33.3%) was observed in layer flocks followed by 30.5% observed in broiler breeders and 4.9% in broilers. It was observed that the detection rate of *MG* and *MS* is higher in Eldakahliya than in Elgarbeyag governorate in layers and breeders and no infection was detected in a layer farm in Giza by the different methods of diagnosis. The lowest detection rate was identified in a broiler farm in Elfayoum.

Moreover, using age-based analyses, the most positive period was 46 weeks, followed by 40 weeks, 34 weeks, 27 weeks, and at least 20 weeks, in order of decreasing seropositivity (Demirbilek et al., 2020). *Mycoplasma synoviae* can spread vertically and horizontally (Stipkovits and Kempf, 1996). It is anticipated that horizontal transmission is the most effective transmission route for infected breeder flocks (Seifi and Shirzad, 2012).

SPA results showed that the highest detection rate of *MS* was found in breeders, followed by commercial layers due to long life span which is in agreement with Seifi and Shirzad (2012) who recorded 47.8% seropositivity by SPA in breeders above 60 weeks of age and also with results of Feberwee et al. (2008) with 60% seropositivity in breeders ≥ 52 weeks of age. It was found that the lowest detection rate was identified in broilers (15%) due to a short life span.SPA tests mainly measure type M immunoglobulin (IgM) which can be detected in serum within a week of infection and persist 70-80 days, while ELISA detects IgG (IgY in birds), which can be detected 7-10 days after infection and persist for up to six months (Bradbury and Morrow, 2008).

El-Jakee et al. (2019) investigated the seroprevalence of *MG* antibodies in 12 broiler breeder flocks and it was 52.92% (634/1198) using ELISA, while in hatched chicks from broiler breeder flocks, the serum plate agglutination test identified antibodies against MG in 52.86% (74/140) of the collected serum samples. The current study results indicated that positive samples were lower with ELISA for *MG* (31.7%) and *MS* (23.6%) than with SPA for *MG* (40%) and *MS* (31.1%) which agrees with Feizi et al. (2013), who recorded 33.33% with ELISA and 42.22% with SPA for *MG* and also with Osman et al. (2009), who recorded 41.9% with ELISA and 54.8% with SPA for *MG* and also with Luciano et al. (2011) who recorded (26.46%) positive in SPA and (4.21%) positive in HI and (21.06%) positive in ELISA and they observed weak statistical relation between all serological tests (SPA, HI, and ELISA). Ali et al. (2015) recorded that of 563 samples, 64.47% and 56.13% showed a complete prevalence of MG antibodies in ELISA and SPA tests respectively.

Despite the lower positivity of ELISA, it gave higher specificity than the SPA test for the detection of specific antibodies (Reda and Elsamie, 2012) as SPA is considered less specific than ELISA but the higher detection of positive birds by SPA may also be attributed to new infections (birds which developed an IgM response but did not have time to develop an IgY response to infection). Indirect ELISA was done by Bari and Shareef (2023) to evaluate the prevalence of MG antibodies in serum samples which were collected from 20 broiler flocks in Duhok governorate and all the serum-positive reactors to MG were 52.48%. The highest prevalence of *Mycoplasma* recorded by serological tests may be attributed to false positive results which can be related to the use of inactivated vaccines, recent infection with different *Mycoplasma* species which leads to cross-reactions, lack of heat inactivation, and age of birds (Feizi et al., 2013). In addition, the presence of antiglobulin-like factors and sera from chickens infected with infectious bursal disease viruses that cross-react in MG SPA tests can result in nonspecific reactions, Moreover, different degrees of temporary immunosuppression might permit a more prominent invasion of MG, and successively positive serological response (Asgharzade et al., 2013).

Using serological tests is recommended by OIE for screening only in flocks' diagnoses and not for individual birds' diagnoses as serological tests are rapid and easily performed. As serology gives information on the positive/negative status of the flock towards MG/MS it does not mean that mycoplasmas are still present in the flock (memory effect of serology, infection that may have happened several months before). Moreover, researchers must not depend on serological tests only for the diagnosis of *Mycoplasma* due to different sensitivities and specificities of serological tests. Isolation of microorganisms by culture method and/or molecular technique as PCR is a must to ensure the diagnosis (OIE, 2008).

By comparing different methods for diagnosis of *Mycoplasma* infection, it was found that the highest detection rate of MG and MS was detected by serological tests followed by PCR, with the lowest prevalence detected by the conventional culture method. Accordingly, both PCR and ELISA methods were considered superior

to the culture method for detecting avian mycoplasmosis (Qasem et al., 2015). Results of TaqMan RT-PCR showed an 81.25% detection rate, whereas the conventional polymerase chain reaction assay detected 51.92% positive cases (Elbehiry et al., 2016).

The lowest prevalence of MG (10.8%) and MS (3.9%) detected by the conventional culture method is probably because the culture of *Mycoplasma* species is fastidious and time-consuming as isolation takes a long time. Moreover, the detection of *Mycoplasma* species in medicated birds and chronic cases is very difficult due to low concentrations of mycoplasmas in these cases and culture is less sensitive than PCR (Gondal et al., 2015). The prevalence of MG by culture (10.8%) is lower than that detected by PCR technique (16.7%) and in correspondence with Gondalet al. (2015) who recorded a lower prevalence for culture (27.3%) than that for PCR detection (49.74%).

PCR is an alternative to the traditional isolation technique (Ferguson et al., 2005; Hess et al., 2007; Evans and Leigh, 2008) as it is more specific than the culture method. This is attributed to the fastidious nature of microorganisms, the high sensitivity of PCR tests, and the capability of PCR to amplify DNA from dead or alive pathogens. Application of molecular methods (PCR) on a large scale is used for accurate diagnosis of avian mycoplasmosis that aids in disease eradication programs to minimize the economic losses in poultry farms (Marouf et al., 2020). PCR is the most sensitive and reliable tool for the diagnosis of avian mycoplasmosis in field samples (Muhammad et al., 2018).

The culture technique is the gold standard test and PCR is a confirmative test but it does not differentiate between dead and live cells. Therefore, Culture must be performed in parallel with PCR to ensure greater diagnostic security.

CONCLUSION

In the present study, the highest detection rate of MG and MS is observed in layers and breeders, respectively while the lowest prevalence for both MG and MS are observed in broilers in Egypt poultry farms. Serological methods and PCR from tracheal samples gave better sensitivity than culture methods and can be used in the diagnosis of avian mycoplasmosis. Future research is recommended to identify the best prevention programs, hygienic measurements, effective treatments, and vaccination for the prevention and control of *M. gallisepticum* and *M. synoviae* in poultry production in Egypt.

DECLARATIONS

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This study received no funding.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

Ethical considerations

The authors considered farmers' ethical concerns and consent before conducting the study. This article was written originally without any copy from other articles.

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Authors contributions

Dr. Marwa Emam collected the data, participated in the design of the work protocol, and performed the laboratory work. Dr. Mahmoud El Hariri and Dr. Yousreya Mohamed Hashem found the research idea, shared the performed data, and designed the work protocol. Dr. Elshaimaa Ismael performed the statistical analysis of the study. Dr. Jakeen El Jakee supervised the findings of the work. All authors discussed the results and contributed to the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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