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Research Article

Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by Real-Time PCRs and *Mycoplasma gallisepticum*-antibody Detection by an ELISA in Chicken Breeder Flocks

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Abstract

This study aimed to determine the prevalence of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in breeder flocks showing respiratory symptoms. A total of 77 flocks (2153 tracheal swabs and blood samples) were sampled and all were tested by MG real time PCR (MG-rPCR) and MG-ELISA, and 32 flocks were tested by MS real time PCR (MS-rPCR). In the first part of this study covering 28 flocks, all samples from chickens with marked clinical symptoms and high MG-antibody levels gave negative results with MG-rPCR$_1$. Therefore, the MG-lipoprotein gene-specific primers (MG-rPCR$_1$) of this PCR were replaced with MG-16S rRNA primers (MG-rPCR$_2$), as were the MS-16S rRNA primers (MS-rPCR), thus the study was pursued accordingly. All of the first 28 flocks, which were 100% positive by MG-ELISA, were MG-rPCR$_1$ negative, whereas in the second part of the study, other 49 flocks, which were 87.8% MG seropositive, were found 42.9% positive by MG-rPCR$_2$. In addition, 5 selected flocks from the first 28 were negative, whereas 7.4% of the 27 selected flocks from the second 49 were positive by MS-rPCR. Overall, 81 out of 432 MG-rPCR$_{1\text{-}2}$ (18.7%) performed from 77 flocks, and 13 out of 187 MS-rPCRs (6.9%) in 32 flocks were determined as positive. ELISA results indicated that there could be significantly high false-positives in serological tests, thus results should not be relied upon one test system. Also, this study revealed that, for the confirmation of *Mycoplasma*-infected flocks in laboratories, rPCR is a reliable method as long as suitable primers are selected, and that MG and MS prevalence is considerably high in winter season.

Keywords: *Mycoplasma gallisepticum, Mycoplasma synoviae, Real time PCR, ELISA, Breeder chicken*

Damızlık Tavuk Kümeslerinde *Mycoplasma gallisepticum*ve *Mycoplasma synoviae*'nin Gerçek Zamanlı PCR'lar ile ve *Mycoplasma gallisepticum* Antikorlarının ELISA ile Tespiti

Özet

Bu çalışmada, *Mycoplasma gallisepticum* (MG) ve *Mycoplasma synoviae* (MS)'nın solunum sistemine ait semptomlara sahip damızlık tavuk kümeslerindeki yaygınlığını tespit etmek amaçlandı. Toplam 77 kümes (2153 trakeal svab ve kan örneği)'in toplanan tüm örnekleri MG gerçek zamanlı PCR (MG-rPCR) ve MG-ELISA ile, 32 kümes ise MS-rPCR ile test edildi. Çalışmanın birinci bölümünü kapsayan ilk 28 kümeste MG-antikorları yüksek bulundu ve belirgin klinik semptomlar gösteren bu tavukların örnekleri MG-rPCR$_1$ ile negatif sonuç verdi. Bu nedenle, bu PCR'da kullanılan MG-lipoprotein geni-spesifik primerler (MG-rPCR$_1$), MS tespitinde kullanılan MS-16S rRNA primerlerine (MS-rPCR) benzer olarak, MG-16S rRNA primerleri (MG-rPCR$_2$) ile değiştirildi ve çalışmaya bu primerler ile devam edildi. MG-ELISA ile %100 pozitif olan ilk 28 kümesin tümü MG-rPCR$_1$ ile negatif iken, çalışmanın 2. bölümünde %87.8 MG seropozitif olarak saptanan diğer 49 kümes MG-rPCR$_2$ ile %42.9 pozitif bulundu. Ayrıca, bu ilk 28 kümesten seçilen 5 adetinde MS-rPCR negatif iken, daha sonraki 49 kümesten seçilen 27'si MS-rPCR ile %7.4 pozitif olarak tespit edildi. Genel olarak, toplam 77 kümeste uygulanan 432 MG-rPCR$_{1\text{-}2}$'den 81'i (%18.7) ve bu kümeslerden 32 adetinde yapılan 187 MS-rPCR'ın 13'ü (%6.9) pozitif bulundu. ELISA sonuçları ise serolojik testlerden önemli oranda yanlış-pozitif sonuçlar alınabileceğini ve tek bir test sistemine güvenilmemesi gerektiğini işaret etti. Bu çalışma ile, aynı zamanda, *Mycoplasma*-enfekte kümeslerin laboratuvarlarda doğrulanabilmesi için doğru primerler seçildiğinde rPCR'ın güvenli bir metot olduğu, damızlık tavuk kümeslerinde MG ve MS prevalansının özellikle kış mevsiminde oldukça yüksek olduğu ortaya kondu.

Anahtar sözcükler: *Mycoplasma gallisepticum, Mycoplasma synoviae, Gerçek zamanlı PCR, ELISA, Damızlık tavuk*

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INTRODUCTION

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are infectious agents of chronic respiratory disease in chickens [1]. Multi-age commercial egg complexes are mostly positive for MG and MS and in some parts of the world, both infections are widespread in commercial chicken and turkey production [2]. MG and MS cause important primary and secondary bacterial poultry diseases [2,3].

Due to the persistent nature of the *Mycoplasma* infection and its vertical mode of transmission, monitoring and eradication is a preferable strategy for the achievement of a long-lasting *Mycoplasma*-free poultry stock [4]. Isolation and identification of the organism is the 'gold standard' for diagnosis of *Mycoplasma* infections. However, pathogenic avian mycoplasmas are slow growing (might require up to 3 weeks for detectable growth) and relatively fastidious organism, which are suppressed by use of antibiotic therapy, and, commonly overgrown by commensals such as *Mycoplasma gallinarum* and *Mycoplasma gallinaceum* [2].

Serology including ELISA is much faster than culturing, but nonspecific reactions and cross-reactions between bacterial species, misinterpretations due to recent vaccination, and high cost are all disadvantage of serology [1,5]. MG strains of low virulence typically produce a poor antibody response, and isolation from clinical specimens may be difficult [6]. Variability in strains and clinical responses were noted both for MG and MS [1,5], and, Feberwee et al.[1] indicated encountering flocks exhibiting low levels of serological response.

PCR is a rapid, sensitive and specific method, and is often used to complement culture to detect the presence of specific *Mycoplasma* DNA. There are different PCR procedures such as conventional and real time PCRs (rPCR) for MG or MS detection and their advantages and disadvantages have previously been discussed [1,4,5,7-10].

The objective of this study was rapid detection of MG and MS using rPCRs with three different primer pairs in the chicken breeder flocks, which were screened for antibody to MG by ELISA during the winter and summer seasons.

MATERIAL and METHODS

Standard MG and MS Strains

The MG S6 and the MS K1858 strains were kindly provided by *Mycoplasma* Laboratory, Pendik Veterinary Research Institute, Istanbul, Turkey, and were used as positive controls.

Tracheal Swap and Blood Samples

During 16 months trial period (including one winter season) a total of 432 live chicken tracheal swab samples, comprised of 2153 individual samples, each pooled into 4-5, from 77 breeder flocks, with no antibiotic treatment, belonging to 13 companies, for MG were sampled as described [11], and transferred to the laboratory for rPCR test. Thirty-two of these 77 breeder flocks' same tracheal swab samples, belonging to 5 of these 13 companies, pooled into 127 tracheal samples, comprised of 935 individual tracheal swab samples, also used for MS-rPCR. That is, 77 breeder flocks were sampled for MG-rPCR and the same templates from 32 of these 77 breeder flocks' samples were also tested for MS-rPCR. All flocks had respiratory symptoms and tracheal swab samples were taken from chickens with marked respiratory symptoms. Concurrently, 2153 blood samples from same chickens were collected and tested by MG specific ELISA.

MG-ELISA

ELISA was performed using *Mycoplasma gallisepticum* antibody test kit (Synbiotics, catalog no: AUCMG900, Zoetis) following the instructions described by the manufacturer.

DNA Extraction

DNA was extracted from pooled tracheal swab samples, suspended and vortexed in 1 ml of sterile PBS in 1.5 µl tubes. The suspension was centrifuged for 30 min at 14.000 x g at 4ºC, and the swabs were discarded. The supernatant was carefully removed and the pellet was resuspended in 25 µl sterile deionized water. Then, this mix was boiled at 95-100ºC for 30 min, and kept on ice for 10 min before centrifugation at 14.000 x g for 5 min. The supernatant was used as template in rPCRs.

Primers

Primers used in this study for detection of MG and MS genes, references, amplified product size, name's of the PCR, number of the PCR worked with these primers, company's cod worked with these primers were shown in *Table 1*.

MG and MSrPCRs

rPCR reactions (MG-rPCR$_1$, MG-rPCR$_2$, MS-rPCR) were performed with the same conditions and cycling parameters by a LightCycler™ 2.0 system (Roche Diagnostics, Mannheim, Germany) using FastStart DNA Master SYBR Green I (SGI) PCR mix and reagents (Roche, catalog no: 03 003 230 001). Each reaction was performed in 20 µl volumes, including 18 µl of reaction mixture containing 2 µl 1X LC FastStart DNA SGI Master Mix, 4 mM $MgCl_2$, 0.5 mM of each primer and 2 µl of template DNA. Cycling parameters were as follows: initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 10 s, annealing at 50ºC for 10 s and extension at 72ºC for 20 s.

Table 1. *Primers used in this study for detection of MG and MS genes, references, amplified product size, name's of the PCR, number of the PCR worked with these primers, company's cod worked with these primers*

Tablo 1. *MG ve MS genlerinin tespiti için çalışmada kullanılan primerler, alındıkları literatürler, primerlerin amplifikasyon büyüklüğü, PCR'ın adı, primerlerle yapılan PCR sayısı, primerlerle çalışılan şirketlerin kodu*

Gene	Sequence (5'-3')	Reference	Amplified Product Size (bp)	Name's of the PCR	Number of the PCR, Worked These Primers	Company's Cod, Worked with These Primers
MG- lipoprotein gene	GATTTCGAAGAATCAACTGT AAGGGATTAATATTCCCAAC	[8]	400	MG-rPCR$_1$	159	1, 2, 3, 4, 5
MG-16S rRNA gene	GAGCTAATCTGTAAAGTTGGTC GCTTCCTTGCGGTTAGCAAC	[12]	185	MG-rPCR$_2$	273	6, 7, 8, 9, 10, 11, 12, 13
MS-16S rRNA gene	GAGAAGCAAAATAGTGATATC TCGTCTCCGAAGTTAACAA	[10]	207	MS-rPCR	187	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13

RESULTS

MG and MS rPCRs

No MG was detected by MG-rPCR$_1$ and MS-rPCR from the 28 and 5 of the flocks, respectively. Number of pooled samples for MG and MS detection and seasonal evaluation were demonstrated in *Table 2*. Flocks were found 42.9% (21/49) and 7.4% (2/27) positive by both MGr-PCR$_2$ and MS-rPCR, respectively. Among pooled samples, 12.8% (35/273) and 10.2% (13/127) were positive by MG-rPCR$_2$ and MS-rPCR, respectively. There was no MGr-PCR$_2$ positivity in summer, whereas this rate increased to 44% positivity in winter. Similarly, MS-rPCR detection rate was 0% in summer opposed to 10.8% positivity in winter *(Table 3)*. Overall, 81 out of 432 (18.7%) samples were positive by MG-rPCR$_{1\text{-}2}$ in 77 flocks, and 13 out of 187 (6.9%) samples were positive by MS-rPCR in 32 flocks. In flock-based evaluation, rPCR positive flock rate was 23.4% (18/77) and 8.1% (2/32) for MG and MS, respectively. Overall, MG-rPCRs were 33% positive during winter and 0% in summer season. MS detection rate was 8.1% and 0% in winter and summer, respectively *(Table 4)*.

MG-ELISA

Seropositive flock rate was 68.8% (53/77) by MG-ELISA. Individually, 453 of the 2153 (21.0%) samples were positive by MG-ELISA. Seroprevalence of MG infection was higher during winter season (27.6%) than in summer season (12.7%) *(Table 4)*.

DISCUSSION

Initial MG-rPCR$_1$ yielding consecutively negative results in contrast to 100% seropositivity in flocks by MG-ELISA from 28 flocks *(Table 2)* was resolved after replacement of MG-lipoprotein primers with MG-16S rRNA primers in the study. Results from this new rPCR, which was designated as MG-rPCR$_2$ were consistent with MG-ELISA results: flocks were found 42.9% (21/49) positive by MG-rPCR$_2$ and 87.8% (43/49) positive by MG-ELISA *(Table 3)*. There are earlier reports on PCR assays targeting the 16S rRNA gene regions [1,9,10], whereas more recent assays attempted to target more species-specific gene regions [4,5,8,13]. rRNA genes are present in all prokaryotes and include regions that are highly conserved among bacteria. Raviv and Kleven have reported [4] that PCR assays target the 16S rRNA gene might cross-react with other mollicutes and prokaryotes. In our study, when we replaced the MG-lipoprotein-based primers with 16S rRNA-based primers, our results started to match up with our serological data, which is in contrast to the findings of Raviv and Kleven [4]. Garcia et al.[13] compared the 16S rRNA, *mgc*2, lipoprotein and *gap*A surface protein genes for MG detection and found that *mgc*2 and the 16S rRNA methods had similar and the best detection limits. Hess et al.[14] reported that MG 16S rRNA gene-based PCR, which was developed by Lauermann [12], had higher analytical sensitivity than other PCR methods tested. It has been reported that the 16S rRNA-based PCR can amplify DNA from *Mycoplasma imitans* (MI), a phylogenetically related avian *Mycoplasma* with very similar

Table 2. *Results of tracheal swab and sera samples of 5 companies (company cod: 1-5) by MG-rPCR$_1$ (with primers of lipoprotein gene), MS-rPCR and MG-ELISA*

Tablo 2. *Beş şirkete ait (şirket kodu: 1-5) trakeal svab ve serum örneklerinin MG-rPCR$_1$ (lipoprotein geni primerleri ile), MS-rPCR ve MG-ELISA sonuçları*

Company	Total Flock/ Positive by MG-rPCR$_1$	Number of Total Tracheal Swap and Sera	Number of Pooled Samples/ MG Positive Samples by MG-rPCR$_1$ - (%)	Total MG-rPCR$_1$/ Positive %		Number of MS-rPCR/ Positive Samples - (%)	Total MS-rPCR/ Positive %		Number of MG-ELISA/ Positive Samples - (%)	Total MG-ELISA Positive Samples %		Season
1	5	80	16/0 - (0)	0 (Summer)	0 (Winter)	-	0 (Summer)	0 (Winter)	80/9 - (11.25)	18.3 (Summer)	16.1 (Winter)	Summer
2	8	200	40/0 - (0)			-			200/17 - (8.5)			Summer
3	6	128	27/0 - (0)			-			128/45 - (35.1)			Summer
4	5	300	60/0 - (0)			60/0 - (0)			300/78 - (26)			Winter
5	4	80	16/0 - (0)			-			80/5 - (6.2)			Winter
Total	28	788	159/0 - (0)			60/0 - (0)			788/154 - (19.5)			

Table 3. *Results of tracheal swab and sera samples of 8 companies (company cod: 6-13) by MG-rPCR$_2$ (with primers of 16S rRNA gene), MS-rPCR and MG-ELISA*
Tablo 3. *Sekiz şirkete ait (şirket kodu: 6-13) trakeal svab ve serum örneklerinin MG-rPCR$_2$ (16S rRNA geni primerleri ile), MS-rPCR ve MG-ELISA sonuçları*

Company	Total Flock/ Positive Flockby MG-rPCR$_2$	Number of Total Tracheal Swap and Sera	Number of Pooled Samples/ MG Positive Samples by rPCR$_2$ - (%)	Total MG-rPCR$_2$/ Positive %		Number of MS-rPCR/ Positive Samples - (%)	Total MS-rPCR/ Positive %		Number of MG-ELISA/ Positive Samples - (%)	Total MG-ELISA Positive Samples %		Season
6	5/5	100	20/16 - (80)	0 (Summer)	44 (Winter)	20/0 - (0)	0 (Summer)	10.8 (Winter)	100/62 - (62)	4,2 (Summer)	31.5 (Winter)	Winter
7	5/5	100	20/20 - (100)			-			100/42 - (42)			Winter
8	3/2	350	70/10 - (14.3)			-			350/35 - (10)			Winter
9	10/7	200	40/35 - (70)			40/0 - (0)			200/103 - (51.5)			Winter
10	6/2^{MS+*}	200	40/0 - (0)			40/13 - (32.5)			200/35 - (17.5)			Winter
11	6	135	27/0 - (0)			27/0 - (0)			135/0 - (0)			Summer
12	10	200	40/0 - (0)			-			200/17 - (8.5)			Summer
13	4	80	16/0 - (0)			-			80/5 - (6.25)			Winter
Total	49/21	1365	273/35 - (12.8)			127/13 - (10.2)			1365/299 - (21.9)			

** 6 flocks of 10. company had no positivity for MG-rPCR$_2$, but 2 flocks had positivity with MS-rPCR*

Table 4. *Total evaluation of worked two MG-PCRs, MS-rPCR and MG-ELISA results*
Tablo 4. *Çalışılan 2 MG-PCRs, MS-rPCR ve MG-ELISA sonuçlarının birlikte değerlendirilmesi*

Total Flock/ Positive by MG-rPCR$_{1-2}$	Total Flock/ Positive by MS-rPCR	Number of Tracheal Swap for MG/MS	Number of Pooled Samples for MG-rPCR$_{1-2}$/ Positive	Total MG-rPCR$_{1-2}$ Positive Samples According to Season %		Number of Pooled Samples for MS-rPCR/ Positive	Total MS-rPCR/ Positive Samples According to Season %		Number of MG-ELISA/ Positive	Total MG-ELISA Positive Samples According to Season %	
77/18	32/2	2153/935	432/81	0 (Summer)	33 (Winter)	187/13	0 (Summer)	8.1 (Winter)	2153/453	12.7 (Summer)	27.6 (Winter)
28%	8.1%		18.7%			6.9%			21.0%		

16S rRNA genes to MG [15]. However, since MI has been only isolated from ducks, geese and partridges, it is considered of limited significance in diagnosis in samples from chickens [16].

We found that the overall rPCR positive flock rate was 23.38% (18/77) and 8.12% (2/32) for MG and MS, respectively *(Table 3)*. In a study from Turkey [5], the MG prevalence in MG positive flocks was found 16.1% (5/31) and 29% (9/31), by MG culture and PCR, respectively. However in other studies in Turkey, the positive results are higher than our results. Cengiz et al.[17] have reported that 14 of 26 (53.8%) investigated flocks were MG-PCR positive. Dakman et al.[18] have found 87.5% MS-PCR positive from 1.200 tracheal swabs and serum samples. Tuzcu et al.[19] have determined 80% MG-PCR positive results from 3 different broiler breeder farms. Aras and Sayın [20] have found that 5 out of 20 (25%) layer flocks were MS positive by PCR. Similarly, the incidence of MS infection detected by PCR in commercial flocks in Brazil and Netherlands has been found as 72.7% and 73%, respectively [7,21]. Also, MG-PCR positive results have been reported as 73.64% and 58.1% in Brazil and Vietnam, respectively [7,22].

In this study, seropositive flock rate was found 68.8% (53/77) by MG-ELISA. Four hundred fifty three individual tracheal samples out of 2153 (21.04%) were positive by MG-ELISA *(Table 4)*. In a previous study [5] MG-seropositive flock rate was reported as 48.4% (15/31) and 32.3% (10/31) by Rapid Plate Agglutination (RPA) and Hem-agglutination Inhibition (HI) tests, respectively. In our study, flocks belonging to companies 1, 2, 3, 4, and 5 had high MG-antibody (6-35%) levels; despite their MG-rPCR$_1$ negative results *(Table 2)*. This could be related either to the possibly lower specificity of the PCR primers used in MG-rPCR$_1$ or to the lower sensitivity of the MG-ELISA. Additionally, we found a high Infectious Bronchitis reverse transcriptase PCR-positive rate [23] from Company 12's same tracheal swab samples (data not shown), which were negative for MG and MS, but MG-antibody rate was 8.5% by MG-ELISA. Feberwee et al.[1] have found that the number of cross-reactions (false positives) in serological tests was lower in infection with the ATCC strains than in infections with the MG and MS field strains. In their study, they compared different commercial ELISA tests for serological identification of MG, and reported that a certain level of false positive results could be expected in any serological test. They also implied that the level of false positive results varied between several serological tests, and concluded that it was not advisable to rely completely on one test (system) only. Likewise, Aras and Sayın [20] indicated that genetic similarity between field MS strains from Turkey could vary 53% to 100% by RAPD analyses. This fact could also have an important impact on the sensitivity and specificity of a particular serological test. In our study, flocks of Companies 6, 7, and 9 had low MG-antibody rate (42-62%) in contrast to their high MG-rPCR$_2$ positivity rates (70-100%) *(Table 3)*. This instance could be explained by the possibility of a recently starting MG infection, which could not yet gave rise to detectable MG-antibody levels. Consequently, we should note here

that serological tests as ELISA are widely used screening methods with known shortcomings as cross reactivity, high sensitivity and low specificity. Therefore, ELISA can only be used in rapid primary screening of the flocks, and should be complemented by culture and PCR for confirmative and definitive diagnosis.

When our results are evaluated on seasonal basis, we found that MG and MS infections were more prevalent especially at winter season than summer season by both PCRs and ELISA *(Table 4)* similar to previous reports [24-26].

In this study, we used two rPCRs with the same cycling parameters and SGI without using specific probes for MG and MS detection primarily to reduce the cost of detection. Secondly, SGI rPCR has considerably higher detection capability than probe-based PCRs, since probes can detect only when all bases match up with the target DNA. Therefore, if there are (point) mutations, as widely seen in field *Mycoplasma* strains, probes may not match up, leading to false negative results. Therefore, SGI rPCR has no match up problem with mutated DNA yielding slightly shifted melting temperatures, which can be determined by melting curve analysis of the PCR product after rPCR.

Previously, the 16S rRNA gene-specific primers used in this study were well studied and evaluated by Lauerman et al.[10] for MS detection, where they found 100% correlation between their MS-PCR on both specificity and sensitivity. When evaluated MG and MS together, two primers sets utilized in this work were previously published with a known sensitivity of 70-100 colony-forming units (CFU) ml^{-1} [10]. Feberwee et al.[1] also reported that culture and PCR tests had comparable sensitivity in detecting both MG and MS with the same primers. Also, Jarquin et al.[9] used these primers in both conventional PCR and SYBR Green I (SGI) rPCR and found that there was no difference between two PCRs. Also, they determined that the SGIrPCR assay developed in their study was more rapid than all three methods tested and more sensitive and specific than culturing or serology. They have found that 13 cases were found positive by PCR and in only 9 cases culture was positive.

Infection of breeder poultry flocks with MG and MS can be economically devastating to producers especially at cold and wet winter seasons. The accurate and the timely diagnosis of MG and MS infections are essential to control these infections in poultry. The rPCR surveillance tool used in this study has the potential to save producers from these large losses on the basis of a reduced detection time, allowing producers to act quickly and prevent spread of disease. Our results showed that suitable primers selected for correct PCR assays with serological method (ELISA) for primary screening of the flocks is a considerably economical approach in diagnosis of MG and MS infections in breeder chicken flocks which are important for providing MG/MS-free progeny.

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