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Original Article
Özgün Araştırma

Development of real-time PCR method for the diagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*

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Abstract: Avian mycoplasmas are associated with respiratory disease, synovitis, poor quality of day-old chicks, and poor performance. The main approach used for the diagnosis of avian mycoplasmas is isolation and identification of the microorganism. Since the *Mycoplasma* are slow-growing fastidious organisms, conventional methods are time-consuming, laborious, and require experienced personnel. For this reason, we aimed to develop a rapid detection method for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) by quantitative real-time polymerase chain reaction (qPCR). For this purpose, the *lipoprotein* (*lp)* and *variable lipoprotein hemagglutinin* (*vlhA)* genes were used to detect *M. gallisepticum* and *M. synoviae*, respectively. The limit of detection (LOD) of the assay was determined to be $<10^1$ DNA/µl from artificially contaminated swab samples. The specificity and sensitivity ratios were detected 100%. Overall, these results indicate that this qPCR method can be accurately used for the detection of MG and MS.

Keywords: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, qPCR

Mycoplasma gallisepticum ve *Mycoplasma synoviae* teşhisi için real-time PCR yöntemi geliştirilmesi

Özet: Kanatlı mikoplazmaları solunum yolu hastalığı, sinovitis, günlük civcivlerin kalitesizliği ve düşük performans ile ilişkilidir. Kanatlı mikoplazmalarının teşhisinde kullanılan temel yaklaşım mikroorganizmanın izolasyonu ve identifikasyonudur. Mikoplazma yavaş ve zor üreyen organizmalar olduğundan, konvansiyonel yöntemler zaman alıcı, zahmetli ve deneyimli personel gerektirir. Bu nedenle, kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qPCR) ile *Mycoplasma gallisepticum* (MG) ve *Mycoplasma synoviae* (MS) için hızlı bir tespit yöntemi geliştirmeyi amaçladık. Bu amaçla, sırasıyla *lipoprotein (lp)* ve *değişken lipoprotein hemaglütinin (vlhA)* genleri *M. gallisepticum* ve *M. synoviae*'nın teşhisi için kullanıldı. Yapay kontaminasyon yapılan svap örneklerinde, geliştirilen metodun deteksiyon limiti (LOD) $<10^1$ DNA/µl olarak belirlendi. Spesifite ve sensitivite oranları ise %100 olarak tespit edildi. Tüm sonuçlar, geliştirilen qPCR metodunun MG ve MS'nın doğru teşhisinde kullanılabilecek bir yöntem olduğunu göstermektedir.

Anahtar kelimeler: *Mycoplasma gallisepticum*, *Mycoplasma synoviae,* qPCR

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are the most important avian *Mycoplasma* species worldwide in the poultry industry (Raviv et al. 2007). MG causes chronic respiratory disease (CRD) of chickens and infectious sinusitis in turkeys. It is characterized by nasal discharges, conjunctivitis, and couching. MG cause also infectious sinusitis in turkeys. MS infection is commonly seen as a subclinical upper respiratory infection. MS may cause air sac lesions when combined with infectious bronchitis (IB) or Newcastle disease (ND). It may also cause infectious synovitis when becomes systematic (Lockaby et al. 1999). MG and MS infections are economically important diseases since they cause decreased egg production, reduced quality of day-old chicks, growth rate, and increased costs of eradication procedures (Ley 2003).

Isolation and identification of *Mycoplasma* species is still considered the "gold standard" method for the diagnosis of the diseases (Kleven et al. 1991; Nascimento et al. 1991). However, replication of *Mycoplasma* species requires a complex medium consisted of serum, 3-5 or longer days, and 2-3 serial passages at 5 to 7-day intervals (Ley 2003). These methods are expensive, time-consuming, and laborious. Serological tests including the rapid slide agglutination test, the hemagglutination inhibition test, and ELISA to detect antibody production are used for the diagnosis of avian mycoplasmosis (Kleven et al. 1991; Yoder 1991). Polymerase chain reaction (PCR) based methods are alternatives for the detection of *Mycoplasma* species. Beside this,

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the culture of the bacteria is not required for the detection by PCR-based methods (Hess et al. 2007; Grodio et al. 2008). Detection of *Mycoplasma* species more rapid and highly specific by quantitative PCR methods has become the frontline approach (Carli and Eyigor 2003; Mekkes and Feberwee 2005; Callison et al. 2006; Grodio et al. 2008).

In this study, a quantitative real-time PCR (qPCR) method was developed for the molecular detection of MG and MS from poultry samples. The developed method was validated by determining the PCR efficiency, the limit of detection (LOD), and specificity tests. This approach was also aimed to screen the presence of MG and MS in poultry samples.

Material and Methods

Genomic DNA of Bacterial strains: The genomic DNA of *M. gallisepticum* and *M. synoviae* strains were obtained from the collection of Ankara University Faculty of Veterinary Medicine, Department of Microbiology.

Bio-Speedy® Universal Real-Time PCR Internal Control Kit, Cat No: BS-AMP-501 (Bioeksen R&D Technologies Ltd, Turkey) was used to monitor the integrity of PCR. For this purpose, 10 µL of the internal control template included in the kit was added to the DNA extract.

Primers and Probes: Detection of *M. gallisepticum* and *M. synoviae* was performed using oligonucleotides targeting the *lipoprotein (lp)* and *variable lipoprotein hemagglutinin (vlhA)* genes, respectively. Probes were designed using Primer3 (v.0.4.0) software with an average length of 18-24 base pairs (bp), 5-8ºC higher than the binding temperatures of forward and reverse primers determined for the same gene region, and GC content not exceeding 60% (in the patent submission process).

qPCR assay: The multiplex qPCR assay was optimized using Quant Studio-5 Real-Time PCR instrument (Applied Biosystems) and the specificity of two primer pairs were confirmed via single PCR. The PCR was optimized to conditions of 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 60°C for 50 s. Amplification of target genes was observed in the FAM and HEX channels for *M. gallisepticum*/*synoviae* and internal control, respectively.

A total of 10 µL of multiplex qPCR mix consisted of 5 µL of Bio-Speedy® Colorless 2X qPCR Mix (Bioeksen R&D Technologies Ltd, Turkey), primers and probes with different concentrations, 2 µL of template nucleic acid to final volume. qPCR reaction setup details are given in Table 1.

The results were recorded as negative if there was no sigmoidal curve. The results were recorded as positive if Cq<37. The analysis was repeated with the same nucleic acid extract if Cq≥37, if the result was Cq≥37 again, the test was repeated from the DNA extraction step.

Table 1. Multiplex qPCR reaction setup details.

Reagent	Final Concentration
Colorless 2X qPCR Mix *(Bio-Speedy®, Cat No: BS-AMP-102)*	1X
Oligomix (MG/MS)	500 nmol/L
Probe (MG/MS)	200 nmol/L
Internal Control Oligo Mix *(Bio-Speedy®, Cat No: BS-AMP-501)*	1 µL
Template Nucleic Acid	2 µL
Final Volume	10 µL

PCR Efficiency: Each *M. gallisepticum* and *M. synoviae* obtained from the collection of Ankara University Faculty of Veterinary Medicine, Department of Microbiology were used for the PCR efficiency tests.

Nucleic acid samples of *M. gallisepticum* and *M. synoviae* were diluted to working concentration (200 ng/µl). A 6-point dilution series of 1/2, 1/4, 1/16, 1/64, 1/256, and 1/1024 were prepared from DNA samples, starting from a concentration of 200 ng/µL. Each dilution was analyzed in duplicate by qPCR. Calibration curves were constructed with the Cq values obtained by the PCR test and the logarithm of the dilution factors. Compliance with the acceptance criteria required for PCR efficiency was evaluated by using the equations of the calibration curves.

Specificity: The specificity was tested wet with a total of 17 microorganisms genomic DNA extraction consisting of *Salmonella* Liverpool, *Salmonella* Kentucky, *Salmonella* Mbandaka, *Salmonella* Agona, *Salmonella* Virchow, *Salmonella* Enteritidis, *Salmonella* Infantis, *Streptococcus pneumoniae, Pseudomonas aeruginosa, Mycoplasma iowae, Mycoplasma meleagridis, Mycoplasma gallisepticum, Mycoplasma synoviae, Pasteurella multocida, Escherichia coli, Staphylococcus aureus, Avibacterium paragallinarum*.

Genomic DNA extraction was performed by using Bio-Speedy® Universal Nucleic Acid Isolation Kit, Cat No: BS-NA-121, and by adding 10 µL of the internal control template included in the Bio-Speedy® Universal Real-Time PCR Internal Control Kit, Cat No: BS-AMP-501 (Bioeksen R&D

Technologies Ltd, Turkey). All samples were tested in triplicate and the Cq values were observed in the related channels.

Limit of detection (LOD): Each *M. gallisepticum* and *M. synoviae* obtained from the collection of Ankara University Faculty of Veterinary Medicine, Department of Microbiology were used in LOD studies.

For the LOD studies, a swab matrix was selected. 6-point dilution series of 1/2, 1/4, 1/16, 1/64, 1/256, and 1/1024 of DNA samples were prepared and swab samples artificially contaminated with target levels. The artificial contamination procedure was performed in five replicates for the swab matrix and each contamination level. In addition, five replicates of negative (uncontaminated) samples were prepared for the swab matrix. All samples were tested in duplicate by two different analysts on different days by qPCR. Positive and negative (no template) control were tested in each run. Nuclease-free water, (DEPC-treated, molecular biology grade, CAS 7732-18-5) was used as a template in negative control reactions.

Results

PCR Efficiency: The qPCR efficiency of *M. gallisepticum* and *M. synoviae* was found to be 98.5% and 98.6%, respectively. The data of the qPCR efficiency tests are given in Table 2.

Specificity: The wet tests showed that the kit does not cross-react with the other strains of the *Mycoplasma* genus or the strains of other microorganisms. In addition, all inclusivity test strains were positive by qPCR (Table 3).

Table 2. Data of PCR efficiency tests.

Target	Dilution Factor	Cq1	Cq2	Cq mean	Cq difference [a] (between previous dilution)
Mycoplasma gallisepticum	1/2	18.32	18.39	18.35	-
	1/4	20.23	20.29	20.26	1.91
	1/16	22.29	22.33	22.306	2.046
	1/64	24.28	24.62	24.445	2.139
	1/256	26.67	26.69	26.68	2.235
	1/1024	28.62	28.71	28.654	1.974
Mycoplasma synoviae	1/2	18.29	18.30	18.3	-
	1/4	20.15	20.37	20.266	1.966
	1/16	22.36	22.38	22.39	2.124
	1/64	24.32	24.35	24.33	1.94
	1/256	26.8	26.85	26.825	2.495
	1/1024	28.75	28.78	28.775	1.95

[a] As the DNA is diluted 4-fold in each dilution, the expected Cq difference with the previous dilution is 2.0 when the PCR efficiency is 100% ($4=2^2$).

Table 3. Selectivity test results of multiplex qPCR with MG/MS and other strains.

Species/Strain [a]	Source [b]	Ip	vlhA	IC
Salmonella enterica subsp. *enterica*				
serovar Enteritidis (1)	AUVFM	-	-	+
serovar Kentucky (1)	AUVFM	-	-	+
serovar Infantis (1)	AUVFM	-	-	+
serovar Mbandaka (1)	AUVFM	-	-	+
serovar Virchow (1)	AUVFM	-	-	+
serovar Liverpool (1)	AUVFM	-	-	+
serovar Agona (1)	AUVFM	-	-	+
Streptococcus pneumoniae (1)	AUVFM	-	-	+
*Escherichia coli (*1)	AUVFM	-	-	+
Pasteurella multocida (1)	AUVFM	-	-	+
Salmonella enterica subsp. *enterica*				
Avibacterium paragallinarum (1)	AUVFM	-	-	+
Pseudomonas aeruginosa (1)	AUVFM	-	-	+
Staphylococcus aureus (1)	AUVFM	-	-	+
Mycoplasma gallisepticum (1)	AUVFM	+	-	+
Mycoplasma synoviae (1)	AUVFM	-	+	+
Mycoplasma iowae (1)	AUVFM	-	-	+
Mycoplasma meleagridis (1)	AUVFM	-	-	+

[a] The numbers in parentheses indicate the number of strains.
[b] AUVFM refers to Ankara University Faculty of Veterinary Medicine Department of Microbiology.

Limit of detection (LOD): The detection limit of the assays was determined to be $<10^1$ DNA/µl from artificially contaminated swab samples. All negative and positive controls tested in the multiplex qPCR runs were eligible.

Discussion

The qPCR method developed in this study detecting the presence of *M. gallisepticum* and *M. synoviae* is essential since these bacteria cause loss of productivity and economic losses in the poultry industry. Although the culture of the *Mycoplasma* species is considered as the gold standard method for the detection of avian *Mycoplasma* species, these methods are time-consuming, labour-intensive, and fastidious (Kleven et al. 1991; Nascimento et al. 1991; Mekkes and Feberwee 2005). For this reason, PCR-based methods have been replaced culture methods for more than a decade (Marois et al. 2002; Mekkes and Feberwee 2005; Fraga et al. 2013; Khalifa et al. 2013; Fujisawa et al. 2019). In this study, the development of a rapid, sensitive and effective method for the detection of *M. gallisepticum* and *M. synoviae* from poultry samples was aimed. Analysis of the qPCR assay resulted in 100% identity for the primer and probe sequences targeting *M. gallisepticum* and *M. synoviae*.

Since the 16S ribosomal DNA (rDNA) gene sequences are highly conserved within the bacteria, for the diagnosis of MG and MS by PCR methods, the 16S rRNA gene was targeted in the early 1990s (Lauerman 1998). However, PCR assays targeted 16S rDNA gene might cross-react with other bacterial species and cause false-negative results (Kempf 1998). Because of this reason we choose PCR primers based on the *lp* gene and *vlhA* gene for detection of MG and MS, respectively (Nascimento et al. 1991; Noormohammadi et al. 2000; Bencina et al. 2001; Carli and Eyigor 2003). The *mgc2* gene of MG and the *vlhA* gene of MS are widely used for the detection of avian Mycoplasma species according to World Organization or Animal Health (OIE). The PCR method targeted to the MG *lp* gene was first described by Nascimento et al. (Nascimento et al. 1991). The *lp* gene was investigated with the *gapA* gene together by nested PCR method by Nascimento et al. Carli and Eyigor (2003) first described the method for the detection of MG in chicken tracheal samples by using qPCR and DNA melting curve analysis. One advantage of the developed method in this study is that targeted the *lp* gene is a single PCR method. For the detection of MS, the *vlhA* gene is already recommended in several studies (Hong et al. 2004; Moscoso et al. 2004; Ghaniei 2016; Fujisawa et al. 2019; Felice et al. 2020).

The detection limit of the assay in this study was found $< 10^1$ DNA/µl from artificially contaminated swab samples. Other studies reported the detection limits in colony-forming units (CFU) and color changing-units (CCU). Carli and Eyigor (2003) reported the value of the LOD assay as 3000 CFU/ml^{-1} for MG. Hong et al. (2004) determined the LOD of the *vlha* PCR assay as 4.7×10^2 CCU/ml. In this context, a direct comparison of detection limits between the studies cannot be made.

The data presented in this study indicate that this qPCR procedure based on the *vlhA* and *lp* genes of *M. synoviae* and *M. gallisepticum* has the favorable sensitivity and specificity required to be useful as a diagnostic PCR. Beside this, the cost of qPCR can be reduced by pooling samples (Khalifa et al. 2013). At present, this developed method has been used in Turkey for the detection of MG and MS in some commercial poultry flocks.

Ethic statement: This study does not present any ethical concerns.

Conflict of Interest Statement: The authors declare no conflicts of interest with respect to the publication of this manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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