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Research Paper

A Multiplex real-time PCR for detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in clinical samples from Brazilian commercial poultry flocks

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Abstract

Mycoplasma gallisepticum (MS) and *Mycoplasma synoviae* (MS) are important avian pathogens and cause economic losses to the poultry industry. Molecular biology techniques are currently used for a rapid detection of these pathogens and the adoption of control measures of the diseases. The aim of this study was to develop and validate a technique for simultaneous detection of MG and MS by multiplex real time polymerase chain reaction (PCR). The complete assay (Multiplex MGMS) was designed with primers and probes specific for each pathogen and developed to be carried out in a single tube reaction. Vaccines, MG and MS isolates and DNA from other *Mycoplasma* species were used for the development and validation of the method. Further, 78 pooled clinical samples from different poultry flocks in Brazil were obtained and used to determine the sensitivity and specificity of the technique in comparison to 2 real time PCR assays specific for MG (MG PCR) and MS (MS PCR). The results demonstrated an agreement of 100% (23 positive and 44 negative samples) between Multiplex MGMS and MG PCR in the analysis of 67 samples from MG positive and negative poultry flocks, and an agreement of 96.9% between Multiplex MGMS and MS PCR in the analysis of 64 samples from MS positive and negative poultry flocks. Considering the single amplification tests as the gold standard, the Multiplex MGMS showed 100% of specificity and sensitivity in the MG analysis and 94.7% sensitivity and 100% specificity in the MS analysis. This new assay could be used for rapid analysis of MG and MS in the poultry industry laboratories.

Key words: real-time PCR, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*.

Introduction

Mycoplasmas are small prokaryotes devoid of cell walls. These bacteria are classified in the genus *Mycoplasma*, belong to the class Mollicutes (division Tenericutes) and are taxonomically characterized by phenotype, serology and sequencing of the 16S rRNA (Brown *et al.*, 2007). Approximately 120 different species of *Mycoplasma* were already identified infecting different organisms, but only 20 are adapted to birds. Two of them, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), are important pathogens causing respiratory disease, synovitis and airsacculitis in commercial poultry. Infection control for MG and MS is essential in all stages of the industry production chain to prevent loss of productivity due to decreased production/quality of the eggs, decreased feed efficiency and high condemnations rates of carcasses (Kleven, 2008a; Nascimento and Pereira, 2009).

MG and MS control programs developed by government departments have been maintained in important producing countries. In Brazil, the National Plan for Avian Health (PNSA, *Plano Nacional de Sanidade Avícola*) recommends several field and laboratory procedures for the effective control of these pathogens in commercial poultry

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flocks. These procedures include regular serological monitoring in the flocks using Rapid Plate Agglutination Test (RPA), ELISA or Haemagglutination Inhibition (HI) (Ewing *et al.*, 1996; Kleven, 2008a). As these routine tests have accuracy limitations, it is required to confirm the results with more specialized procedures, as bacterial isolation and Polymerase Chain Reaction (PCR) assay.

Isolation and identification of the bacteria is still considered the "gold standard" for diagnosis of *Mycoplasma* infections (Kleven, 2008b; Nascimento and Pereira, 2009). However mycoplasmas are slow growing relatively fastidious organisms. Further, it is common the development of other commensal bacteria of the same genus (as *M. gallinarum* and *M. gallinaceum*) in the routine procedures for isolation of MG and MS (Kleven, 2008a). Consequently PCR assay has been increasingly used as a valuable alternative test for the direct detection of MG and MS in clinical samples and several different procedures were described in scientific literature over the past decade (García *et al.*, 2005; Hess *et al.*, 2007; Lauerman *et al.*, 1993; Nascimento *et al.*, 1991; Silveira *et al.*, 1996). Recently, real-time PCR assays were developed for a faster and easier diagnostic of these pathogens in comparison to the conventional PCR assays (Callison *et al.*, 2006; Mekkes and Feberwee, 2005; Raviv and Kleven, 2009). Another important advantage of this technique is the possibility of simultaneous detection of 2 or more pathogens in a single assay (Multiplex PCR assay), reducing labor, time and costs for the complete analysis (Sprygin *et al.*, 2010).

This study aimed to develop and validate a methodology for the simultaneous detection of the *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by a real time Multiplex PCR (Multiplex MGMS).

Materials and Methods

Mycoplasma DNA

DNA extracted from 1 isolate of MG, 14 different MS isolates (WVU-1853, F10-2AS, MS-H, K4463, 00-10650, 00-10651, 00-10652, 00-10653, 00-10654, 00-10655, 00-10656, 00-10657, 00-10658, 00-10659), 11 other species of *Mycoplasma* (*M. meleagridis, M. imitants, M. pullorum, M. gallinarum, M. gallinaceum, M. cloacale, M. iners, M. gallopavonis, M. iowae, M. lipofasciens, M. columbinum*) and one *Acholeplasma* were kindly provided by Dr. Maricarmen García (University of Georgia, USA). Further 80 stored DNA extracts from poultry clinical samples, previously analyzed by PCR for MS, were separated for the validation assays of the real time PCR for MS.

Vaccine and field samples

MG and MS live vaccines marketed in Brazil were obtained from different suppliers: strain F (F Vax-MG® from Intervet - Schering Plough Animal Health and *Mycoplasma gallisepticum* vaccine® from Lohmann Animal Health), 6/85 (Mycovac-L® from Intervet - Schering Plough Animal Health), ts-11 (MG ts-11® from Merial), MG-70 (Myco-Galli® from Biovet) and MS-H (Mycovax® from Merial). A total of 78 field samples (trachea or tracheal swabs) were obtained from different commercial poultry flocks (broilers, layers and breeders) in Brazil during the period of February 2010 to January 2011. All flocks were suspected to be infected with MG or MS, most of them with positive or indeterminate results in serological tests (RPA, ELISA or HI).

DNA extraction

Total DNA of the clinical samples and vaccines was purified by a standard silica/GuSCN-based procedure (Boom *et al.*, 1990) using a commercial kit (Newgene, Simbios Biotecnologia). Briefly, all swabs samples were re-suspended on 2.5 mL of lysis buffer (GuSCN 5 M, Tris-HCl 0.1 M, EDTA 0.5 M and Triton X-100) and then incubated at 56 °C for 10 min. For the liquid samples (vaccines), 100 µL was mixed to 400 µL of lysis buffer and incubated at 56 °C for 10 min. After, 500 µL was aliquoted in a separate tube and 20 µL of silica suspension was added and mixed. Tubes were submitted to centrifugation at 8600 g for 30 s. The pellet was washed once with 500 µL washing buffer (GuSCN 5 M and Tris-HCl 0.1 M), once with 150 µL washing buffer, twice with 150 µL 75% ethanol and once with 150 µL of ethanol absolute. Silica suspension was dried at 56-60 °C for 15 min. DNA was eluted with 50 µL of TE buffer and incubated at 60 °C for 5 min, and the solution was separated of the silica particles centrifuging at 8600 g for 3 min.

DNA amplification

PCR was performed in a thermocycler StepOnePlus™ Real Time PCR System (Applied Biosystems, USA) using specific primers and probes for each pathogen. Primers and probes used for detection of MG were previously described (Callison *et al.*, 2006). Primers used for the real time PCR MS assay were also previously published (Lauerman *et al.*, 1993), but the probe was designed in this study (Table 1).

Three separate reactions (MG, MS and Multiplex MGMS) were carried out independently. MG and MS real time PCR assays were performed in a total volume of 30 µL containing 3 µL of 10x PCR buffer, 23 µL of H_20 treated, 0.9 µL of $MgCl_2$ (50 mM), 0.08 µL of deoxynucleotide triphosphate (2.1 mM), 0.04 µL of the each primer (50 µM) and probe (25 µM), 0.6 µL of ROX, 0.32 µL of Taq DNA polymerase (5 U/µL) and 2 µL of DNA template. The reaction was carried out in a thermocycle program with an initial denaturing step of 95 °C for 3 min and 40 cycles of 95 °C for 30 s, followed by annealing/extension at 60 °C for 60 s.

Table 1 - Primers and probes used in Multiplex MGMS.

	Target gene	Primers and probes
MG#	Lipoprotein	MGLPU26-F 5' - CTA GAG GGT TGG ACA GTT ATG - 3'
		MGLP164-R 5' - GCT GCA CTA AAT GAT ACG TCA AA - 3'
		MGLP-P 5' - (FAM) - CAG TCA TTA ACA ACT TAC CAC CAG AAT CTG - (MGB) - 3'
MS*	16SrRNA	MS1 5' - GAA GCA AAA TAG TGA TAT CA - 3'
		MS2 5' - GTC GTC TCC GAA GTT AAC AA - 3'
		MS-P 5- (VIC) - AGC TAC GCT ACG GTG AAT ACG TTC TC - (TAMRA) - 3

#Primers and probe published by Calisson *et al.*, 2006.
*Primers designed for Lauerman *et al.*, 1993, and the probe designed in this study.

All the multiplex reactions (MGMS) were run in a total volume of 30 μL with 2 μL of template DNA, 21.1 μL of DEPC treated H_20, 3 μL of specific buffer, 0.9 μL of $MgCl_2$ (50 mM), 0.9 μL of deoxynucleotide triphosphate (2.1 mM), 0.15 μL of primers for MG (final concentration of 0.25 μM for each one in the reaction) e 0.3 μL of primers for MS, 0.15 μL of respective probe (final concentration of 0.25 μM), 0.3 μL of *Taq* DNA polymerase (5 U/μL) (Cenbiot Enzimas, RS, Brazil) and 0.6 μL of ROX. The reaction was carried out in a thermocycle program with the same temperature and time conditions of the MG and MS real time PCR assays. Samples with any recorded threshold cycle number (Ct) value were considered positive and samples with no recorded Ct value were considered negative. Amplicons were also submitted to electrophoresis in polyacrilamide gels to confirm the amplification of the specific 139-base pair (bp) fragment length for MG and 207-bp fragment for MS.

Field performance of the multiplex assay

Agreement between tests was evaluated by Kappa test with a confidence level of 95% (Win Episcope 2.0). Sensitivity and specificity of the test was determined using field samples results. The analytical specificity of the test was calculated using the formula: true negative (TN) / TN + false positive (FP). The analytical sensitivity was calculated as follows: true positive (TP) / TP + false negative (FN). Therefore, TN were samples negative by both compared assays; FP were samples negative by PCR MG and MS and positive by Multiplex MGMS; TP were samples positive by both compared assays; FN were samples positive by PCR MG and MS and negative by Multiplex MGMS.

Results

Real time PCR MG and MS assay

Real-time PCR for MG was performed exactly as previously published (Callison *et al.*, 2006). DNA extract of the only MG isolate and 4 vaccine strains presented positive Cts (varying from 20.8 to 23.0). Further, DNA extracts from the other 11 different *Mycoplasma* species, 1 *Acholeplasma* and 14 MS isolates of MS and the MS-H vaccine strain did not present any Ct signal (Table 2). DNA extracted from 5 dilutions of the MG-70 vaccine strain presented a very good linear correlation wit the respective Ct values. Analytical specificity/sensitivity and detection limit were assumed to be the same of the previous work (Callison *et al.*, 2006).

MS real time PCR assay was set up using the same reaction conditions of the MG real time PCR assay, as described in the Materials and Methods. All the 14 isolates of MS tested for MS real time PCR presented a positive Ct (varying from 17.6 to 39.0). DNA extracts from the 10 different *Mycoplasma* species, 1 *Acholeplasma* and 4 MG vaccine strains did not present any Ct signal. *M. cloacale* showed cross reaction with Ct of 29.0 (Table 2).

The limit of detection was evaluated using DNA extracted from 6 five-fold dilutions of the vaccine strain MS-H (Mycovax®). Two replicates of each dilution were subjected to the MS real time PCR assay and to another previously published PCR test, defined as Lauerman PCR (Lauerman *et al.*, 1993). In the MS real time PCR analysis, positive signal was obtained with the undiluted sample (Ct 17.6) and 5 of the 6 dilutions (Ct means of 20.7, 23.8, 26.4, 28.6, and 33.9, respectively). All the samples with readable Ct presented a specific band of 207 bp in the Lauerman test.

Performance of the MS real time PCR assay was determined using 80 DNA extracts obtained of field samples and previously analyzed by Lauerman PCR. A total of 77 results were concordant between the 2 assays (43 positive and 34 negative) and only 3 results were discordant (positive by Lauerman PCR and negative by MS real time PCR assay). Sensitivity and specificity for the MS real time PCR assay were 93.9% and 100%, respectively.

Multiplex validation tests

Based on the successful results of the MG and MS real time assays, a novel detection procedure based on PCR Multiplex (Multiplex MGMS) was designed. The whole procedure consisted in a unique amplification procedure with the two MG and MS primers pairs and respective probes.

Optimization of the Multiplex MGMS was performed with 3 independent runs using dilutions of 25 and 625-fold dilution from a vaccine strain for MG (Myco-Galli®, strain MG-70, Biovet) and MS (Mycovax®, strain MS-H, Merial). All the dilutions were subjected to PCR amplification by the specific real time PCR for each species (MG and MS) and Multiplex MGMS. Three replicates were carried out to determine the mean Ct values for each template.

The results showed a strong relationship between the dilutions of MG and MS DNAs (vaccine strains MG-70 and MS-H, respectively) and the value of cycle threshold, where the lowest Ct value corresponds to the sample of higher concentration for both MG and MS (Table 3). An excelent linear correlation was observed between DNA dilutions and the respective Ct values (0.98 for MS and 1.00 for MG), comparable to the real time PCR assays for MS (0.96) and MG (1.00). Further, all the positive samples for MS were not detected with the MG probe and all positive samples for MG were not detected with the MS probe (Table 3).

The specificity of the Multiplex MGMS was verified by testing DNA extracts from different *Mollicutes*, including *M. gallisepticum* and *M. synoviae*. No other *Mycoplasma* species was detected by Multiplex MGMS, with the exception of *M. cloacale* who presented a positive signal for MS primers/probe set. This same sample has also presented positive result using the MS real time PCR assay (Table 2). All MG and MS strains tested were readily detected in the Multiplex MGMS and in the specific PCR for MG and MS. The Multiplex MGMS was able to detect *M. gallisepticum* and *M. synoviae* independently and simultaneously.

Field performance of the multiplex MGMS

To evaluate the correlation between the Multiplex MGMS and MG and MS separate real time PCR assays, pools of tracheal swabs collected from commercial flocks were tested by the 3 assays. From the 78 sample collected, a total of 53 were positive in the Multiplex MGMS, 23 for MG and 36 for MS (6 samples were positive for MG and

Table 2 - Specificity of Multiplex MGMS assay compared with the specific test for MG and MS by analyzing different mycoplasmas.

	Multiplex MGMS		Specific test	
Organism	MS	MG	MS	MG
Acholeplasma	Negative	Negative	Negative	Negative
M. cloacale	29.0	Negative	34.4	Negative
M. columbinsale	Negative	Negative	Negative	Negative
M. gallinaceum	Negative	Negative	Negative	Negative
M. gallinarum	Negative	Negative	Negative	Negative
M. gallopavonis	Negative	Negative	Negative	Negative
M. imitans	Negative	Negative	Negative	Negative
M. iners	Negative	Negative	Negative	Negative
M. iowae	Negative	Negative	Negative	Negative
M. lipofaciens	Negative	Negative	Negative	Negative
M. meleagridis	Negative	Negative	Negative	Negative
M. pullorum	Negative	Negative	Negative	Negative
M. synoviae	22.2	Negative	21.3	Negative
M. gallisepticum	Negative	21.5	Negative	20.8

Table 3 - Comparison between the dilutions of the vaccine strains (for MG and MS) and the Ct values obtained in three replicates.

	Concentration	Multiplex PCR		Specific PCR	
		MS Ct# (DP*)	MG Ct# (DP*)	MS Ct# (DP*)	MG Ct# (DP*)
MS	High	22.0 (0.1)	Negative	21.6 (0.2)	Negative
	Intermediate	27.0 (0.4)	Negative	26.5 (0.4)	Negative
	Low	35.4 (0.8)	Negative	37.1 (0.8)	Negative
MG	High	Negative	22.0 (0.1)	Negative	22.1 (0.1)
	Intermediate	Negative	26.5 (0.3)	Negative	26.7 (0.3)
	Low	Negative	32.0 (0.5)	Negative	31.5 (0.3)

#Average Ct of the three replicates.
*Standard deviation.

Table 4 - Analysis of the sensitivity and specificity of the Multiplex PCR MGMS compared to specific real time PCR MG and MS.

	Specific PCR							
	MS (n = 64)				MG (n = 67)			
Multiplex PCR MGMS		+	-	Total	MG	+	-	Total
	+	36	0	36	+	23	0	23
	-	2	26	28	-	0	44	44

In agreement analysis using the Kappa test results for MS and MG were, respectively: 0.936 and 1.

MS). From these samples, 67 were also analyzed by MG PCR assay and 64 by MS PCR assay. The comparative results are presented in Table 4. In the analysis of MG, all samples were concordant and consequently with 100% of sensitivity and specificity. In the analysis of MS, there were 2 discordant cases (both positive for MS real time PCR assay and negative for Multiplex MGMS), with values of sensitivity of 94.7% and a specificity of 100%.

Discussion

In the present study, a new Multiplex real time Taqman™ PCR assay was developed and applied for simultaneous detection of *M. gallisepticum* and *M. synoviae* in poultry clinical samples. For the development of this assay, we first implemented a MS real time PCR test, based on the combination of a previously extensively tested primers set (Lauerman *et al.*, 1993) and a newly designed internal Taqman probe. This assay was validated in comparison with a largely used PCR assay (Lauerman *et al.*, 1993). After we implemented a previously developed MG real time PCR assay (named MGLP) in our laboratory (Callison *et al.*, 2006). These both procedures presented very good performances and have been widely used in the Brazilian poultry industry laboratories in the last years. Finally, both real time PCR tests were combined for the development of a Multiplex real time PCR assay (Multiplex MGMS).

In the validation tests of the Multiplex MGMS, results showed an excelent linear correlation between the DNA dilutions (of MG and MS) and the Ct value. MG and MS were also detected independently and simultaneously by the PCR Multiplex MGMS, with no interference of increasing MG DNA concentration in the MS analysis and vice-versa (data not shown). Further, samples with low DNA concentration (625-fold vaccine dilutions) presented a readable Ct, showing a good analytical sensitivity of the new assay. In the specificity assay, almost all other *Mycoplasma* species were not detected by the Multiplex MGMS assay. The only exception was a cross reaction (with readable Ct of 29.0) in the MS primers/probe set in the analysis of the *M. cloacale* DNA. However, this result was also observed in the MS real time PCR assay (Ct 34.4). *M. cloacale* is a bacteria originally isolated from geese in Europe and supposed to be associated with inflammation of the cloaca in these birds (Stipkovits *et al.*, 1984, 1986). This species was also found in wild North American waterfowl, but it was not associated to any disease (Goldberg *et al.*, 1995). There is not description of *M. cloacale* in commercial poultry flocks in Brazil or other regions of the world.

The Multiplex MGMS presented a very good performance under field conditions. In the comparison with the real time PCR MG and MS assays, specificity values of 100% were obtained for both pathogens and sensitivity values of 100% and 94.7% were obtained for MG and MS, respectively. This lower sensitivity in the MS analysis was because 2 discordant results (positive in the MS real time PCR assay and negative in the Multiplex MGMS) obtained in the analysis of 2 stored samples that were submitted to freezing and thawing. These 2 samples presented high Ct values (29.7 and 29.6), probably associated to a low number of bacteria in the original samples as previously observed (Sprygin *et al.*, 2010). All the analysis performed with fresh samples presented 100% of correlation between the results (including samples with high Cts) (data not shown).

The success of MG and MS control programs depends on accurate and fast diagnostic techniques. Other previous studies had already demonstrated that real time PCR assays (including multiplex procedures) are effective tools for the sensitive and specific detection of these pathogens in poultry commercial flocks (Callison *et al.*, 2006, Sprygin *et al.*, 2010, Raviv and Kleven, 2009; Jarquin *et al.*, 2009). The present work describes a new multiplex PCR for the simultaneous detection of the 2 main *Mycoplasma* pathogens that occurs in Brazilian commercial poultry. This new assay was able to amplify these 2 pathogens in a same analytical procedure and in only one amplification round, saving time and reducing costs in the diagnostic laboratory. Incorporation of this methodology in the poultry industry laboratories would certainly improve the *Mycoplasma* control programs.

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