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Real-time PCR culture and serology for the diagnosis of *Mycoplasma gallisepticum* in chicken breeder flocks

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ABSTRACT

This study aimed to compare a real-time PCR (rPCR) test with improved detection limit to serology and culture for the detection of *Mycoplasma gallisepticum* (MG) infection in chicken breeder flocks. Six hundred and forty-six blood and tracheal swab samples belonging to 31 grandparent chicken breeder flocks were tested by rPCR. The detection limit of rPCR was 0.9 pg μl^{-1}, with pure MG S6 strain DNA and 100 colony forming units (CFUs) ml^{-1}, where both pure culture and tracheal swabs were artificially spiked with the same strain.

The seropositive flock rate based on both MG RPA and HI were calculated as 48.4% (15/31) and 32.3% (10/31), respectively, while culture- and rPCR-positive flock rates were 16.1% (5/31) and 29.0% (9/31), respectively. On flock-based analysis, culture method detected 5 out of 10 MG seropositive flocks (sensitivity 50%, specificity 100%), whereas rPCR detected 8 out of 10 flocks (sensitivity 80%, specificity 95%). Agreements between serology and culture, and serology and rPCR were 83.9% and 90.3%, respectively. On individual sample-based analysis, culture method detected 26 out of 78 MG seropositive chicken (sensitivity 33%, specificity 100%), whereas rPCR detected 51 out of 78 MG seropositive chickens (sensitivity 65%, specificity 96%). There was 91.9% and 91.4% agreement between serology and culture, and serology and rPCR, respectively.

Results of this study indicate that the rPCR with improved in vitro detection limit could detect MG in seropositive chicken flocks. Therefore, we advise the use of rPCR and/or culture for confirmation of serology results obtained from screening MG infection in chicken flocks.

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1. Introduction

Mycoplasma gallisepticum (MG) infections cause significant economic losses in poultry production. Chronic respiratory disease in chickens caused by MG can be diagnosed based on clinical and pathological findings as well as with other tests/methods, including detection of specific antibodies and isolation of MG or detection of specific DNA by PCR (Garcia et al., 1995; Kleven, 2008). For detection of MG infection in flocks, they are screened by serological tests, such as rapid serum agglutination (RSA), hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) (Anonymous, 2004, 2008; Kempf, 1998). *Mycoplasma* culture and PCR are referred as confirmation tests when there is inconclusive serology result (Anonymous, 2004, 2008; Kleven, 1997), which can be related to seroconversion lagging behind infection (Kleven, 1975), non-specific reactions due to cross-reactions with

Abbreviations: CFU, colony forming unit; ELISA, enzyme-linked immunosorbent assay; HI, hemagglutination Inhibition; MG, *Mycoplasma gallisepticum*; NPIP, National Poultry Improvement Plan; OIE, Office International Epizootie; RSA, rapid serum agglutination; PCR, polymerase chain reaction; rPCR, real-time PCR.

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other pathogenic mycoplasmas (Kempf et al., 1997; Kleven et al., 1996), and use of oil emulsion vaccines (Glisson et al., 1984). In some instances, difficulties were reported to detect atypical MG strains by culture, which also give low serological response (Kempf et al., 1997).

MG culture is time-consuming and can require 4–30 days; furthermore, it is unproductive at times, due to overgrowth by other bacteria or because of the inhibitory effect from previous antibiotic therapy (Kempf et al., 1997; Mekkes and Feberwee, 2005; Salisch et al., 1998). Indirect fluorescent antibody tests or indirect immunoperoxidase tests, usually carried out to identify *Mycoplasma* species, both require expertise and are relatively expensive; therefore, they are restricted to specialised laboratories (Salisch et al., 1998). Recently, OIE: Office International Epizootie (OIE, 2) and National Poultry Improvement Plan (NPIP, 1) recommended PCR as a reliable test for the detection of MG infections. Real-time PCR (rPCR), which has distinct advantages over conventional PCR, such as higher reliability, rapidity and prevention of environmental contamination, has been used for the detection and quantification of MG in poultry (Carli and Eyigor, 2003; Hess et al., 2007; Mekkes and Feberwee, 2005). Additionally, two recent studies (Evans and Leigh, 2008; Raviv et al., 2008) addressed the successful differentiation of MG vaccine and wild type strains by rPCR tests. Previously, we had reported a disagreement in our MG rPCR study, whereby several chicken tracheal samples tested as MG-seropositive, but were rPCR-negative and had suspected that this problem arose from the use of a relatively low quality template DNA (Carli and Eyigor, 2003). To solve this problem, in this study, we used a commercial DNA isolation kit, and obtained a better quality template DNA, resulting a 10-fold increase in assay sensitivity with artificially spiked tracheal swab samples.

In this study, we compared this rPCR test with improved detection limit to serology and culture for the detection of MG infection in chicken breeder flocks.

2. Materials and methods

2.1. Standard MG strains and HI test antigen

The MG S6 strain and the standardized HI test antigen containing the MG S6 strain were kindly provided by Mycoplasma Laboratory, Pendik Veterinary Research Institute, Istanbul, Turkey. Other *Mycoplasma* spp. cultures were kindly provided by Dr. Stanley Kleven, Department of Avian Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA (Table 1). MG S6 strain DNA was used during PCR optimisations and as a positive control with clinical sample DNAs for PCR.

2.2. Clinical samples

Tracheal swabs from 646 live chickens belonging to 31 breeder grandparent flocks, with no antibiotic treatment, were sampled as described (Zain and Bradbury, 1995) and transferred to the laboratory for culture and rPCR tests. One tracheal swab per bird was used both for rPCR and culture as follows: First, the tracheal swab was streaked onto Frey's agar (BBL, Becton-Dickinson, No. 211456), and then placed into 500 μl sterile physiologic saline. After vortexing for 2 min, a 100 μl aliquot including the swab was transferred into Frey's broth (BBL, Becton-Dickinson, No. 212346) for culture as described in Section 2.10. The remaining aliquot of 400 μl was used in DNA isolation. In addition, blood samples from the same 646 chickens were taken for RSA and HI at the same time of tracheal sampling (Table 2).

Table 1
Mycoplasma gallisepticum and related strains used in this study.

Strain no	Strain name
1	*Mycoplasma gallisepticum* S6
2	*Mycoplasma gallisepticum* A5969
3	*Mycoplasma gallisepticum* K781(R)
4	*Mycoplasma gallisepticum* K810(F)
5	*Mycoplasma gallisepticum* K3254(6/85)
6	*Mycoplasma gallisepticum* K4548(HF51)
7	*Mycoplasma gallisepticum* K4957F(2)
8	*Mycoplasma gallisepticum* K4958F(2)
9	*Mycoplasma gallisepticum* K4962C(2)
10	*Mycoplasma gallisepticum* Ts-11
11	*Mycoplasma synoviae* 1823(WVU)
12	*Mycoplasma synoviae* K1415(F10-2A5)
13	*Mycoplasma synoviae* K3904(F10-2A5)
14	*Mycoplasma synoviae* K4910(9)
15	*Mycoplasma synoviae* K4927C
16	*Mycoplasma synoviae* K4933D
17	*Mycoplasma gallinarum* K285-"B"-LPG16
18	*Mycoplasma gallinaceum* Tulley "DD"
19	*Mycoplasma gallopavonis* SA (377)
20	*Mycoplasma meleagridis* E-2 (386)
21	*Mycoplasma iowae* K3761(1) 22p (863)
22	*Mycoplasma pullorum* D2403 (396)
23	*Mycoplasma iners* K285-"E"-PG30 (19)
24	*Mycoplasma columbinasale* Bx63 (15)
25	*Mycoplasma lipofaciens* Bx101 (40)
26	*Mycoplasma cloacale* Bx101 (48)
27	*Acholeplasma laidlawii* Bx250 (28)

2.3. DNA extraction

DNAs from MG strains, other avian *Mycplasma* species and clinical samples were extracted with a commercial DNA isolation kit (QIAMP DNA mini kit, 51304, Germany).

2.4. Primers

Forward and reverse PCR primers, MG1 (GATTTCGAAGAATCAACTGT) and MG2 (AAGGGATTAATATTCCCAAC), were selected from a region within the sequence of MG lipoprotein gene partial codons; this primer set had a previously determined sensitivity and specificity for MG, and the expected amplicon size was 400 bp (Carli and Eyigor, 2003).

2.5. rPCR

rPCR was performed using a LightCycler™ 2.0 (Roche Diagnostics, Mannheim, Germany). Each reaction had a volume of 20 μl, including 18 μl of reaction mixture containing 1× LC FastStart DNA SYBR Green I Master Mix (Roche), $MgCl_2$ (4 mM), a 0.5 μM concentration of each primer and 2 μl of template DNA. Cycling parameters were

Table 2
Serology, culture and rPCR results from flocks tested.

Flock no	Age of flock (week)	No. RSA positive/no. tested[a]	No. HI positive (suspicious)[b]/ no. tested[c]	No. culture positive/ no. tested[d]	No rPCR-positive/ no. tested[e]
1	24	7/15	0[f] (2)/15	0/15	0/15
2	40	5/10	0 (3)/10	0/10	0/10
3	16	0/16	0/16	0/16	0/16
4	NA[g]	0/20	0/20	0/20	0/20
5	61	0/10	0/10	0/10	0/10
6	44	0/15	0/15	0/15	0/15
7	32	14/14	7/14	2/14	9/14
8	NA	0/21	0/21	0/21	0/21
9	23	11/13	0 (2)/13	0/13	0/13
10	NA	0/25	0/25	0/25	0/25
11	17	13/14	3 (1)/14	1/14	0/14
12	NA	0/11	0/11	0/11	0/11
13	24	10/10	4/10	0/10	4/10
14	NA	0/11	0/11	0/11	0/11
15	30	5/10	0/10	0/10	0/10
16	NA	9/19	6/19	0/19	2/19
17	NA	0/33	0/33	0/33	0/33
18	40	0/30	0/30	0/30	0/30
19	42	20/20	9/20	8/20	16/20
20	10	41/50	15/50	0/50	3/50
21	21	0/20	0/20	0/20	0/20
22	22	6/10	2/10	1/10	3/10
23	20	0/10	0/10	0/10	1/10
24	20	32/37	9/37	0/37	3/37
25	22	0/16	0/16	0/16	0/16
26	60	37/48	21/48	14/48	32/48
27	30	7/10	0/10	0/10	0/10
28	19	10/12	2 (1)/12	0/12	0/12
29	55	0/30	0/30	0/30	0/30
30	20	0/15	0/15	0/15	0/15
31	29	0/71	0/71	0/71	0/71
Total		227/646	78 (9)/646	26/646	73/646

[a] Number of rapid serum agglutination test-positive samples/number of blood samples tested.
[b] Number of MG suspicious samples with titres of 1/40 based on a hemagglutination inhibition test.
[c] Number of hemagglutination inhibition test-positive samples/number of blood samples tested.
[d] Number of culture-positive samples/number of tracheal swabs tested.
[e] Number of rPCR-positive samples/number of tracheal swabs tested.
[f] Samples considered negative with titres below 1/40 based on a hemagglutination inhibition test.
[g] NA: not available.

as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 50 °C for 5 s and extension at 72 °C for 20 s. Melting curve analysis was performed automatically by LightCycler 2.0 software (Version 3), and the melting peaks were expected to occur at a melting temperature (T_m) of 80 °C.

2.6. Detection limit of rPCR with pure MG DNA

Ten-fold dilutions were prepared from MG S6 standard culture DNA with an initial concentration of 9.2 ng/μl and 2 μl from each dilution were used as a template in rPCR.

2.7. Detection limit of rPCR with pure MG culture

Initially, mean MG S6 strain cell counts in the stock culture were determined by triple plating of 100 μl from 10-fold dilutions of Frey's broth stock onto Frey's agar. After this, 10-fold dilutions of stock MG S6 strain culture, with an average initial concentration of 10^6 colony forming units (CFUs) ml^{-1}, were prepared in Frey's broth down to 1 CFU ml^{-1}. Each dilution was re-diluted 10-fold by taking 100 μl from each broth and transferring it to 900 μl of sterile physiologic saline. DNAs from each suspension were then isolated with the QIAMP DNA mini kit (QIAGEN 51304, Germany), stored at −20 °C and used as a template in rPCRs.

2.8. Detection limit of rPCR with artificially spiked samples

Tracheal swabs spiked artificially with the MG S6 strain were used for this purpose. Briefly, 100 μl of 10-fold dilutions from 10^6 to 1 CFU ml^{-1} of the MG S6 strain were mixed with individual tracheal swabs from MG-seronegative chickens in Eppendorf tubes in 900 μl of sterile physiologic saline and were vortexed for 2 min. The swabs were then discarded and DNA isolations were performed on the suspensions using the QIAMP DNA mini kit. Two microliters from these DNAs were used as a template in rPCR.

2.9. Specificity of rPCR

Specificity of rPCR was tested with the DNAs from a panel of standard MG cultures and related avian *Mycoplasma* species (Table 1).

2.10. Mycoplasma culture

Swabbing from trachea of the birds was performed as described by Zain and Bradbury (1995) and the isolation of MG from these tracheal swabs was done following the standard culture method described by Kleven (2008). Briefly, the tracheal swabs were first streaked onto Frey's agar and the plates were incubated at 37 °C with 5% CO_2 in high humidity. One hundred microliters aliquot from the vortexed 500 μl sterile physiologic saline, containing the tracheal swab was transferred into Frey's broth and incubated at 37 °C with 5% CO_2 in high humidity. Colour change in Frey's broth was observed every day, and the change in colour from pink to orange-yellow in the tube was considered as positive. One week after incubation, cultured broths with colours unchanged were transferred into new Frey's broth, followed by one more passage if the colour was unchanged after 1-week incubation. Positive broths were streaked onto Frey's agar and incubated at 37 °C with 5% CO_2 in high humidity. Plates were checked for typical colonies under an inverted microscope for at least 2 weeks. Possible *Mycoplasma* colonies on plates were confirmed as MG by colony PCR. MG S6 culture was used as a positive control during culture to assure the testing efficacy of the media used.

2.11. Serology

RSA and HI tests were applied to fresh serum samples in order to detect anti-MG antibodies (Anonymous, 2004). MG antigen solution (Nobilis, Intervet International B.V., Holland) was used in RSA following the manufacturer's instructions. Briefly, 20 μl of undiluted serum was mixed with 20 μl of MG antigen and incubated at room temperature for 2 min. When agglutination occurred, the samples were additionally tested in the MG HI test. The HI test was performed according to OIE (Anonymous, 2008). The HI titre was determined and defined as the highest serum dilution exhibiting complete inhibition of HA. HI titres of 1/80 or above and titres of 1/40 are considered positive and strongly suspicious, respectively. Positive and negative control sera were incorporated into each RSA and HI test. 'Seropositivity of the flock or the sample' was defined as 'the flock or the sample positive by HI tests', since this test is the confirmatory test after RSA.

2.12. Statistical analysis

Agreement analyses between serology (Gold Standard) and culture, and serology and rPCR was performed in SPSS 17.00 by applying McNemar Test. Sensitivity and specificity results are calculated accordingly.

3. Results

3.1. Detection limit of rPCR with pure MG DNA

The detection limit of rPCR with pure MG S6 DNA was 0.9 pg μl^{-1}.

3.2. Detection limit of rPCR with pure MG culture

The sensitivity of rPCR with a pure culture of the MG S6 strain was 100 CFU ml^{-1}.

3.3. Detection limit of rPCR with artificially spiked samples

The sensitivity of rPCR with samples spiked artificially with MG S6 culture was 100 CFU ml^{-1}.

3.4. Specificity of rPCR

rPCR yielded specific amplification and T_m peaks of 78.39–79.96 °C with all standard MG strains tested. No peak was observed in the PCRs of non-*M. gallisepticum* strains.

3.5. Flock-based evaluation of serology, culture and rPCR results

As seen in Table 2, the seropositive flock rate was 32.3% (10/31), while culture- and rPCR-positive flock rates were 16.1% (5/31) and 29.0% (9/31), respectively. One seropositive flock (Flock number 28) was determined as negative by culture and rPCR. One flock (Flock number 23) tested positive by rPCR, but negative by serology and culture. All culture-positive flocks but one (Flock number 11, which is rPCR-negative) were positive in serology and rPCR. When rPCR was compared with culture, five flocks (Flock numbers 13, 16, 20, 23 and 24) were determined as positive by rPCR, but were negative by culture. There were five seropositive/culture-negative flocks (Flock numbers 13, 16, 20, 24 and 28); however, no culture-positive/seronegative flock was encountered. A total of four flocks, Flock numbers 7, 19, 22 and 26, were positive by serology, culture and rPCR (Table 2). On flock-based analysis, culture method detected 5 out of 10 MG seropositive flocks (sensitivity 50%, specificity 100%), whereas rPCR detected 8 out of 10 flocks (sensitivity 80%, specificity 95%) (Table 3). There was 83.9% [(5 + 21)/31] agreement between serology and culture, while a 90.3% [(8 + 20)/31] agreement was observed between serology and rPCR.

3.6. Individual sample-based assessment of serology, culture and rPCR results

Seventy-eight and 568 of 646 chickens were found seropositive and seronegative, respectively (Table 2). Tracheal swab samples from 568 seronegative chickens were also culture- and rPCR-negative, except one sample in Flock 23, which was positive by rPCR. Twenty-six and 72 tracheal swab samples from 78 specific MG positive chickens were positive by culture and rPCR, respectively. A total of 25 tracheal swab samples – two from Flock number 7, eight from Flock number 19, one from Flock number 22 and 14 from Flock number 26 – were positive by both culture and rPCR (Table 2). On individual sample-based analysis, culture method detected 26 out of 78 MG seropositive chicken (sensitivity 33%, specificity 100%), whereas rPCR detected 51 out of 78 MG seropositive chickens (sensitivity 65%, specificity 96%) (Table 3). There

Table 3
Flock and individual sample-based agreement results between serology, culture and rPCR.

	Serology		Significance	Sensitivity (%)	Specificity (%)
	Positive	Negative			
Flock-based analysis					
Culture					
Positive	26	0	$P < 0.001$	33	100
Negative	52	568			
rPCR					
Positive	51	22	$P = 0.658$	65	96
Negative	27	546			
Individual sample-based analysis					
Culture					
Positive	5	0	$P = 0.063$	50	100
Negative	5	21			
rPCR					
Positive	8	1	$P = 1.00$	80	95
Negative	2	20			

was 91.9% [(26 + 568)/646] agreement between serology and culture, while a 91.4% [(51 + 546)/646] agreement was observed between serology and rPCR.

4. Discussion

In our previous report, obtaining all MG negative PCR results from 68 seropositive chicken tracheal swabs led us to conclude that this test would not be applicable for tracheal samples from subclinically infected live birds, although the test gave 18 positive results out of 28 samples taken from mucosal surface of the trachea of dead or necropsized birds, indicating that the test worked relatively well (Carli and Eyigor, 2003). We suspected this problem in the detection of MG DNA from live birds of our PCR assay (Carli and Eyigor, 2003), which was also noted by Callison et al. (2006), could be related to the use of low quality template DNA. Therefore, in this study, we used a commercial DNA isolation kit, which enabled us to obtain a better quality template DNA and increased the sensitivity of the rPCR assay by 10-fold in artificially spiked tracheal swab samples, which was also supported by a 0.9 pg μl^{-1} detection limit with pure MG S6 DNA. Use of similar isolation kits to obtain good quality template DNA for PCR has also been reported in other studies (Callison et al., 2006; Raviv and Kleven, 2009). Still, there are previous MG-PCR studies (Lauerman, 1998; Mekkes and Feberwee, 2005), which reported wash-boiling-centrifugation methods for MG DNA template preparation and did not mention any detection problems from tracheal samples related to this issue.

This study showed that undiluted RPA is not suitable in serodiagnosis for MG and that a more specific test like HI is needed. Further on the 5 suspect serological results based on both RPA and HI test in flocks, can be either explained by the beginning of an infection in the flock or by aspecific reactions. The seropositivity in chickens with rPCR-negative results (Flock number 28), which might be due to non-specific agglutinations related to oil-adjuvanted killed vaccines at ages of 17–19 weeks (Table 2), was confirmed more reliably by evaluating HI results, while only one sample in Flock 11 was determined as rPCR-negative but culture positive. This PCR negativity can be explained by the presence of inhibitory factors in the reaction or lack of specific template DNA. Other reasons for the slightly higher positivity in serology as compared to rPCR might be due to non-specific agglutinations derived from non-pathogenic *Mycoplasma* strains or the presence of other pathogenic *Mycoplasma* species in the chickens (Avakian et al., 1988; Glisson et al., 1984). For this type of non-specific positive or suspicious results, which cannot be resolved by HI or ELISA, we think that application of culture and/or PCR as supplementary tests would be beneficial, as indicated in NPIP and OIE (Anonymous, 2004, 2008).

We have observed that the number of positive flocks and individual samples detected by rPCR was more than those determined by culture. Explanations that may address such problems with culture, which were reported previously, include: (1) MG is difficult to grow in culture because of its inherently slow growth and overgrowth by other saprophytic mycoplasmas that may be present in swab samples; rPCR would not be affected by such competition and thus is probably much more sensitive than culture (Callison et al., 2006; Raviv and Kleven, 2009). (2) rPCR assays can amplify nucleic acid from non-viable MG in vitro as well as in vivo (Kempf, 1998). Nevertheless, the presence of MG DNA in the swab sample indicates the recent presence of the organism and can consequently be interpreted as the detection of infected chickens. Interestingly, we found one flock (Number 23) that was positive by rPCR, but was serology- and culture-negative. No isolation of MG in PCR-positive cases had also previously been reported (Hill, 1995; Marois et al., 2002; Salisch et al., 1998). In this study, this flock (Number 23) could have been infected with MG very recently, and the chickens in this flock would not have had enough time to produce sufficient amounts of detectable antibodies. In addition, there were possibly not enough MG cells to detect by culture. Another explanation is for this result may be related to the high sensitivity of our rPCR test, which could necessitate retesting of the sample by rPCR to eliminate a false-positivity. All other culture-positive flocks were diagnosed as positive by serology and rPCR, as expected.

We found relatively low sensitivity of culture and rPCR as 50% and 80%, respectively, compared to serology for determining MG seropositive flocks. Therefore, we recommend rPCR or rPCR in combination with culture only to be used to confirm MG serology suspect flocks, but not to screen for direct MG diagnosis in flocks without serology. The same low sensitivity problems of culture and rPCR on individual sample-based analysis were also observed, although specificity rates of these two tests were very high, supporting the data of flock-based analysis.

We advise the use of rPCR and/or culture for confirmation of serology results obtained from screening MG infection in chicken flocks.

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