COMPARISON OF DIFFERENT MOLECULAR METHODS FOR DETECTION OF MYCOPLASMA GALLISEPTICUM

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Abstract

An “In house” PCR method was compared with three commercial PCR kits for the detection of Mycoplasma gallisepticum (MG) in cultures grown in a modified Frey broth or agar, in vaccines, as well as in tracheal swabs of SPF chickens infected experimentally. The studied methods showed different specificity (poor specificity of commercial A test) and sensitivity. The “in house” method appeared to be more sensitive (7 fg/µL) than the rest of the tests (70 fg/µL). The “in house” PCR method could differentiate between TS-11 and 6/85 vaccine strains, also in the combination with restriction enzyme length polymorphism (RFLP). Furthermore, distinguishing between TS-11 and pathogenic field strains was also possible. To detect MG in tracheal swabs, SPF chickens were inoculated intranasally with 1x10⁶ colony forming unit (cfu)/mL of ATCC reference MG strain. Tracheal samples were collected 4, 7, 14, 28 and 35 d post infection (d.p.i) and examined with PCR-based methods. The MG strain was detected for the longest time, up to 28 d.p.i., by the “in house” method and commercial kit B, with a stronger result being obtained by the “in house” method. The results showed the usefulness of the studied methods for the MG direct detection in chicken swabs; however, some discrepancies were noted with regard to their different specificity and sensitivity.

Key words: Mycoplasma gallisepticum, PCR, strain differentiation.

Mycoplasma gallisepticum (MG) infections are among the major problems in the poultry industry worldwide, causing chronic diseases in chickens and turkeys (13). MG infection has a wide spectrum of clinical forms, of which chronic respiratory disease of chickens and sinusitis of turkeys are the most significant. Infectivity, tissue tropism, and pathogenicity differ significantly among MG strains, but factors, which influence those characters are still unknown (21). Significant advances in the control of MG infections have been achieved since the introduction of MG vaccines and good biosecurity. However, re-emergence of the disease has been observed recently (15).

Various diagnostic methods have been described for the confirmation of the presence of mycoplasma infection (10). Conventional monitoring scheme for the detection of antibody against MG relies on serological tests, even though there are problems with their sensitivity and specificity (11). Mycoplasmas are known to exhibit a high degree of phenotypic variation (20). Changes of several MG proteins have been described, including putative cytadhesin protein pvpA, which is recognised by the chicken immune system (1, 12). Cultivation techniques used for mycoplasmas are laborious, expensive, and time-consuming, and therefore far from a routine procedure (25). Problems experienced with culture include overgrowth by faster growing Mycoplasma species or other bacteria or suppression of growth by antimicrobial treatments administered to the birds (5). As a consequence, the polymerase chain reaction (PCR) has been regarded as a valuable tool for the diagnosis of mycoplasmas (8, 17).

The goal of the present study was to evaluate and compare conventional “in house” PCR method and commercial PCR kits for MG detection.

Material and Methods

Mycoplasma strains and genome isolation. Fourteen Mycoplasma strains were included in the study: four reference (Mycoplasma gallisepticum ATCC®19610, Mycoplasma synoviae ATCC®25204, Mycoplasma meleagridis ATCC®25294, Mycoplasma iowae ATCC®33552), two vaccinal strains (Mycoplasma Gallisepticum Vaccine, strain TS-11 (Select Laboratories, USA), Nobilis MG 6/85 (Intervet, the Netherlands)) as well as eight international (S6/72) and Polish (MG/69, III/72, MG/76, MG/84, MG/86, 477/05, 35/06 – the description of an isolate includes number designed in laboratory in the first section and year of isolation in the second one) field isolates. DNA was extracted from the cultures grown in modified Frey broth or agar at 37°C (reference strains and field isolates) or from vaccinal lyophilisates resolved in PBS.
buffer (vaccines). Commercial QIAamp DNA Mini Kit (Qiagen) was used for the isolation of bacterial DNA.

**PCR amplification. “In house” methods.** The 20 μl of reaction mixture contained 2 μl of extracted DNA, 0.5 mM each dATP, dGTP, dCTP, dTTP, 1xPCR buffer, 1.5 or 2.5 mM of MgCl₂, and 1.5 or 2.0 U of Taq polymerase and 0.1 mM of Mg2-2R/Mgc2-2F or 0.3 mM of MS-1/MS-2 primers, respectively, for MG or MS fragment gene amplification. For MG genome detection, the method aimed at mgc2 gene according to the method by Lysnyansky et al. (16) was used. Additionally, mgc2-PCR – restriction fragment length polymorphism (RFLP) assay was done. The digestion of the PCR amplicon with enzyme HaeII (New England BioLabs) was carried out according to the manufacturer’s instructions.

**Commercial methods.** Three commercial PCR kits (A, B, C) according to the manufacturer’s protocols were used for MG detection.

**Sensitivity and specificity of PCR.** The sensitivity of the PCR was established by ten-fold diluting of the known DNA amount of MG vaccinal strains TS-11 and 6/85 (2.0 and 7.0 ng/μL, respectively) or MG reference ATCC strain (1x10⁶ colony forming units (cfu)/mL). The highest dilution with positive PCR signal was determined by all “in house” and commercial MG-PCR methods. To evaluate the specificity of the method, the three other species of mycoplasma (Mycoplasma synoviae-MS, Mycoplasma meleagridis-MM and Mycoplasma iowae-MI) were used.

**Experimental design.** Two groups of five 4-week-old SPF chickens (Valo-Lohmann, Germany) kept in isolation were inoculated intranasally: one group with 1.0 ml of Frey broth containing around 1x10⁶ cfu/mL of MG reference ATCC strain and another one served as the negative control. Trachea swabs were individually taken 4, 7, 14, 21, 28 and 35 d post infection (d.p.i.). The trachea samples from each time-point were pooled in 2 ml of Frey broth and stored at -20°C for analysis.

**Results**

Using Mg2-2R/Mgc2-2F primers in the “in house” PCR method, two different bands were obtained: MG field isolates and vaccinal TS-11 strain produced the characteristic amplicons of about 300 base pair (bp) and vaccinal 6/85 strain yielded a PCR product of 237 bp. The sensitivity of this PCR was established at 7 fg/μL of DNA from vaccinal 6/85 strain and approximately 3 x higher, at 20 fg/μL of DNA, of vaccinal TS-11 strain (Fig. 1). No cross-reaction was found with other species of mycoplasmas used in the study (MS, MM, MI).

After the digestion with HaeII, two fragments of approximately 270 and 30 bp were observed only in the case of the TS-11 vaccinal strain (Fig. 2, lanes 7-8, 10); no digestion of PCR products was detected in the case of field MG isolates (Fig. 2, lanes 1 to 6).

A comparison of the sensitivity of all MG-PCR methods for 6/85 vaccine strain are shown in Table 1. The most sensitive proved to be the “in house” PCR method. The test A was the less sensitive, moreover specificity studies of primers used in this methods revealed that they did not react with 6/85 vaccine strain. The very weak band only with undiluted extracted 6/85 DNA (7 ng/μL) was observed. In contrast, some reaction with MM was seen.

**Fig. 1.** Electrophoresis of the sensitivity studies of “in house” MG-PCR. Lanes 1 to 9 - ten-fold dilutions of the known DNA amount of vaccinal 6/85 strain (a) and vaccinal TS-11 strain (b), lane M - DNA size marker 100bp DNA ladder (Fermentas, Lithuania).

**Fig. 2.** Electrophoresis of the “in house” MG-PCR products after digestion with the HaeII restriction enzyme. Lanes 1 to 6 - MG field isolates from 1969-86, lanes 7 to 8 - MG field isolates from 2006, lane 9 - vaccinal strain 6/85, lane 10 - vaccinal strain TS-11, lane M - DNA size marker 100bp DNA ladder (Fermentas, Lithuania).
The “in house” test based on cross-react with other mycoplasmas (commercial kit A), moreover they did not react with the 6/85 genome (4, 15, 22). The results of the comparison of the methods targeted to 16S rRNA, mgc2, gapA, and lipoprotein revealed that the most sensitive was gapA method, which detected four coloring units (ccu)/reaction, less sensitive was the lipoprotein method (400 ccu), and the rest was estimated at 40 ccu (4). There are many different ways for the sensitivity estimation. The sensitivity with regard to the number of viable cells could base on the ccu or cfu. Although the culture can theoretically detect a single organism, in practice the efficacy of an organism growing is much lower and also may vary among different strains (18). Additionally, the cultures of a microorganism could contain some portion of non-viable cells, which are detected by PCR (16). Thus, the sensitivity estimation based on DNA amount is greater than those related to the viability of cells. Our experiment confirmed the less sensitivity of the “in house” method for TS-11 detection than 6/85. Similar results were observed by Lysnyansky et al. (16) and this could be attributed to some differences in mgc2 gene sequences.

The advantage of the mgc2 gene-based “in house” method is the possibility to differentiate between pathogenic field strains and vaccine strains by combining with RFLP. The results of our experiment indicated that the “recent” studied Polish isolates from 2006 are TS-11 vaccine strains in contrary to the “early” Polish ones from 1973-1986 (amplicons not digested by HaeII). Moreover, the differentiation between vaccine strains 6/85 and TS-11 is also possible because these two strains give amplicons of different sizes. The third MG vaccine used e.g. in USA, based on F strain, is not available in Poland, so this method seems to be very valuable. Various methods have been described for strain differentiation, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), or sequencing of the part of genome (4, 15, 22). The results of the mgc2-PCR-RFLP method could be obtained during one-two days directly from DNA extracted from tracheal swab samples in contrary to other methods, which require MG isolation in pure culture, and are costly and time consuming.

All applied PCR-based methods proved to be useful for the MG detection directly in chicken swabs. However, some discrepancies were also noted. The MG strain was detected for the longest time, up to 28 d.p.i., by the “in house” method and commercial kit B, with the strongest result being obtained by the “in house” method. The result obtained by commercial kit C was

### Table 1
Comparison of sensitivity of the examined MG-PCR methods

<table>
<thead>
<tr>
<th>Commercial kits</th>
<th>“in house” MG-PCR</th>
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<tbody>
<tr>
<td>A 4 7 14 21 28 35</td>
<td>A 4 7 14 21 28 35</td>
</tr>
<tr>
<td>B 4 7 14 21 28 35</td>
<td>B 4 7 14 21 28 35</td>
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<tr>
<td>C 4 7 14 21 28 35</td>
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Grey areas – positive tests results

Table 2 shows the results of four MG-PCR methods (three commercial and one “in house”) performed on tracheal swabs. By two MG-PCR methods (one commercial B kit and one “in house”), the positive results were obtained up to the 28th d.p.i. However, the strongest amplifications were observed in the case of the “in house” method. At the same d.p.i., other commercial MG-PCR (kit C) gave the doubtful result. The third commercial MG-PCR test (kit A) detected MG shortly - up to 21 d.p.i.

### Table 2
Comparison of three commercial MG-PCR kits (A, B, C) and “in house” method for the detection of MG in tracheal swabs of infected chickens

<table>
<thead>
<tr>
<th>MG-PCR methods</th>
<th>d.p.i.</th>
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<tbody>
<tr>
<td>A 4 7 14 21 28 35</td>
<td>A 4 7 14 21 28 35</td>
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<tr>
<td>B 4 7 14 21 28 35</td>
<td>B 4 7 14 21 28 35</td>
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<td>C 4 7 14 21 28 35</td>
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Grey areas – positive tests results

### Discussion

There are many different PCR methods applied for MG detection including commercial kits e.g. produced by IDEXX Laboratories, Genekam Biotechnology AG, and others. The sensitivity and specificity of PCR-based methods are mainly dependent on the sequences of chosen primers. The PCR methods used for MG detection are based on the amplification of different gene fragments, mostly the 16S rRNA gene, while other PCR assays amplify other MG genes as pvpA, gapA, lipoprotein, mgc2, and more recently IRS (3, 4, 7, 9, 15, 16, 17, 19, 23). The 16S rRNA is a highly conserved region with low levels of genetic variation, which reduces the likelihood of detection lack of some MG strains (24). However, recent studies demonstrated that MG and Mycoplasma imitans (MIM) had very similar 16S rRNA and PCR method aimed at this region could amplify both organisms (6). Our experimental results revealed that some of the commercial kits could cross-react with other mycoplasmas (commercial kit A with MM), moreover they did not react with the 6/85 vaccine strain (also kit A). From all the studied PCR methods, the “in house” test based on mgc2 gene amplification was the most sensitive (7 pg/µL). The comparison of the methods targeted to 16S rRNA, mgc2, gapA, and lipoprotein revealed that the most sensitive was gapA method, which detected four colour changing units (ccu)/reaction, less sensitive was the lipoprotein method (400 ccu), and the rest was estimated at 40 ccu (4).
doubtful (very weak band) on the 28th d.p.i. Commercial test A detected MG in the shortest time – only up to 21 d.p.i. Although the level of positive results varied between studied PCR based methods, they seem to be very valuable due to their rapidity and relatively low costs. On the other hand, there are possibilities to miss some atypical MG or obtain false positive result with MIM, which could also infect chickens (2, 9).

The four PCR methods evaluated in this study were able to detect MG strains with different specificity (the lowest commercial kit A) and sensitivity (the highest “in house” method). More and more test kits become available on the market and are used by an increasing number of laboratories. It should be however, underlined that even partial validation of these methods using field isolates and conducted under specific conditions of each laboratory could give the information about their usefulness.

References


