COMPARISON OF DIFFERENT MOLECULAR METHODS FOR DETECTION OF MYCOPLASMA SYNOVIAE

KATARZYNA DOMAŃSKA-BLICHARZ, GRZEGORZ TOMCZYK, AND ZENON MINTA
Department of Poultry Diseases, National Veterinary Research Institute, 24-100 Pulawy, Poland
domanska@piwet.pulawy.pl

Received for publication March 10, 2009

Abstract

The “in house” PCR method and four commercial PCR kits (designed symbolically as A, B, C, and D) for the detection of Mycoplasma synoviae (MS) in tracheal swabs of infected experimentally SPF chickens were compared. The chickens were inoculated intranasally with 1x10^4 colour change unit (cfu)/mL of ATCC reference MS strain, and at 4, 7, 14, 28, and 35 d post infection (d.p.i.), the tracheal samples were collected and examined. The methods showed different sensitivities. The commercial test C seemed to be less sensitive (0.5 ng/mL) than the rest of the methods (50 pg/mL). Only the “in house” method and commercial test D detected MS DNA from 7 to 14 d.p.i.; no satisfactory results were obtained with the other kits. Since the methods have shown different sensitivity, their suitability for MS detection seems to be limited.

Key words: chickens, Mycoplasma synoviae, PCR.

Mycoplasma synoviae (MS) infections are economically important problems in poultry worldwide, affecting chickens and turkeys and causing respiratory diseases and, in heavy birds, synovitis and arthritis as well (4). According to the latest issue of the OIE Terrestrial Manual 2008, the disease caused by MS is among the diseases to be notified to the OIE unlike in the previous issue where only Mycoplasma gallisepticum (MG) infection was (13). As MS could be egg-transmitted, a very significant way of controlling MS infections is to maintain MS-free breeder flocks. There are some antibiotics active against MS, which could help to decrease the transmission and to lessen the manifestation of the disease. Significant advances in the control of mycoplasmosis have been achieved since the introduction of MG vaccines and, recently, live attenuated MS vaccine have also appeared (MSH) (10).

In MS infections, diagnosis by different methods have been described (11). Among serological tests, the serum plate agglutination (SPA) and ELISA are commonly used, but both have shown some false-positive activity (7). The SPA test missed infections of commercial layers and breeder flocks, which were detected by ELISA (6). Mycoplasmas are known to exhibit a high degree of phenotypic variation (16). Antigenic variation caused by the recombination between pseudogenes and the haemagglutinin encoding vlhA gene could be the reason for the delayed serologic response in chickens infected with MS (3, 5). The cultivation techniques used for mycoplasmas are laborious, expensive, and time-consuming, and therefore far from a routine procedure (19). Fiorentin et al. (8) studied different diagnostic procedures used for the detection of MS infections and they found that PCR is more sensitive than serology and culture techniques (8).

The goal of the present study was to evaluate and compare conventional “in house” PCR methods and commercial PCR kits for the detection of the MS strain in swabs taken from experimentally infected chickens.

Material and Methods

Mycoplasma strains and genome isolation. Six mycoplasma strains were included in the study: four reference (Mycoplasma gallisepticum ATCC® 19610, Mycoplasma synoviae ATCC® 25204, Mycoplasma meleagrisis ATCC® 25294, Mycoplasma iowae ATCC® 33552) and two vaccinal MG strains (Mycoplasma Gallisepticum Vaccine, strain TS-11 (Select Laboratories, USA), Nobilis MG 6/85 (Intervet, Holand)). DNA was extracted from the cultures grown in a modified Frey broth or agar at 37°C (reference strains and field isolates) or from vaccinal lyophylisates resolved in PBS (vaccines). Commercial QIAamp DNA Mini Kit (Qiagen) for the isolation of bacterial DNA was used.

PCR amplification. “In house” methods. The 20 µl of reaction mixture contained 2 µl of extracted DNA, 0.5 mM each of dATP, dGTP, dCTP, and dTTP, 1xPCR buffer, 1.5 or 2.5 mM of MgCl2, 1.5 or 2.0 U of Taq polymerase and 0.3 mM of MS-1/MS-2 primers.
For MS genome detection, the PCR aimed for the 16S rRNA gene according to the method by Lauerman et al. (12).

**Commercial methods.** Four commercial PCR kits, designed symbolically as A, B, C, and D, were used for MS detection according to the manufacturer’s instructions.

**Sensitivity and specificity of PCR.** The sensitivity of the PCR was established by diluting tenfold (5.0 ng/mL) the known DNA amount of MS reference ATCC strain. The highest dilution with a positive PCR signal was determined by all the “in house” and commercial MS-PCR methods. To evaluate the specificity of the method, three additional mycoplasma reference strains (MG, *Mycoplasma meleagridis*, and *Mycoplasma iowae*) were used.

**Experimental design.** Two groups of 4-week-old SPF chickens (Valo-Lohmann, Germany) kept in isolation were used: one group of five birds inoculated intranasally with 1.0 ml of Frey broth containing around 1x10⁴ cfu/mL of MS reference ATCC strain, and a second group of three birds served as the negative control. Trachea swabs were individually taken at 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**

The MS-PCR “in house” method and the four commercial tests exhibited positive results in the form of a sharp band on agarose gels, a visible dot on a nylon membrane or an amplification plot in the DNA dilution containing 50 pg/mL. The sensitivity of commercial test C was lower because the characteristic amplicon was observed in the dilution containing 0.5 ng/mL (Fig. 1). No cross reaction was found with any other species of mycoplasmas used in the study.

![Fig. 1](image)
Table 1
Comparison of three commercial MS-PCR kits (designated as A, B, C) and the “in house” method for the detection of MS in the tracheal swabs of infected chickens

<table>
<thead>
<tr>
<th>MS-PCR methods</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>“in house”</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Gray areas – positive tests results

Table 1 shows the results of the five MS-PCR methods (four commercial and “in house”) performed on the tracheal swabs collected from the chickens 4, 7, 14, 21, 28, and 35 d.p.i. In three commercial tests based on conventional PCR no satisfactory positive results were obtained in any of the studied time points (the very weak band obtained by one commercial B kit at 28 d.p.i. was probably unspecific). Evidence of the MS DNA presence was observed in the case of the “in house” method from 4 to 21 d.p.i. (the bands at 4 and 21 d.p.i. were very weak) and in the case of commercial kit D from 7 to 14 d.p.i.

Discussion

This study provides a comparison between different PCR-based methods, including commercial kits e.g. those produced by IDEXX Laboratories, Genekam Biotechnology AG, Adiagene and the “in house” method for the detection of MS strains. The performance of these tests was determined on samples containing two-fold dilutions of MS ATCC strain, but also derived from experimentally infected 4-week-old SPF chickens. The sensitivity and specificity of the PCR-based methods are mainly dependent on the sequences of chosen primers. The PCR methods used for MS detection are based on the amplification of various gene fragments; the most popular is the 16S rRNA, while other PCR assays amplify such MS genes as vlhA and the fragment located between the intergenic spacer region (IRS) and the 23S rRNA gene (10, 12, 15). The 16S rRNA is a highly conserved region with low levels of genetic variation, which reduces the likelihood of the detection of the lack of some MS (18). On the other hand, 16S rRNA PCR methods could also amplify the DNA of other species of Mycoplasma. It is known that two phylogenetically related avian mycoplasmas, MG and Mycoplasma imitans, have very similar 16S RNA genes and that the primers targeted at this gene give similar products (9). However, M. imitans infects mainly duck, goose, and partridges and so have a limited significance in the samples from chickens (1). Due to the fact that there are two copies of this gene on the MG and MS genomes, the PCR targeted 16S rRNA may have a higher sensitivity (14, 17). The genome sequencing of different avian mycoplasmas revealed that there is a sequence fragment, the IRS located between 16S and 23S rRNA, which has greater inter-species variation than in the 16S rRNA gene, mainly due to the fewer evolutionary constraints on this region (2, 9). The use of the ISR as the target for the detection of MS has been recently described (15). The sensitivity of this method was estimated to 1 ng of DNA/mL and was comparable to the sensitivity of the commercial kits used in our study. It should be mentioned that the methods used for the sample preparation have a great significance on the detection of the results. Ramirez et al. (15) found that some of the applied DNA extraction method allowed the detection of DNA at a dilution of $10^{-2}$ and the second one at a dilution 100-times lower (15).

From the PCR-based methods applied, the most useful for the detection of MS in chicken swabs was the “in house” method and the commercial kit D detecting MS DNA in the real time model. The presence of the MS strain was detected by the “in house” method from 4 to 21 d.p.i.; however, the result obtained in time-point 4 d.p.i. and 21 d.p.i. was doubtful (a very weak band). The commercial kit D detected MS DNA from 7 to 14 d.p.i. with a Ct value of around 30-32 (with all 45 cycles in PCR). Surprisingly, no results were obtained by the rest of the tested commercial kits (A, B, and C). The reason for this may be connected with the differences in their sensitivity, but more probably with the susceptibility of the reagents included in the kits to the presence of some interfering substances/inhibitors. Although the level of positive results varied between the studied PCR based methods, they seem to be very valuable due to their speed and relatively low costs. The real time PCR, due to the removal of the post-PCR detection procedures, and the use of fluorogenic probes, which increased its specificity, appeared to be very useful.

The five PCR methods evaluated in this study were able to detect the MS strains with different sensitivities (the most sensitive were the “in house” method and commercial kit D). More and more test kits are becoming available on the market, and are used by an increasing number of laboratories. But it should be underlined that even the partially validation of these methods, with a specific focus on the method used for the preparation of the sample, could provide information about their usefulness.
References


