Comparison of Antibodies Detection Time with Rapid Plate Agglutination (RPA) Test and with Enzyme-linked Immunosorbent Assay (ELISA) in Mycoplasma gallisepticum (MG) Infections

Avian mycoplasma is a cosmopolitan disease whose economic impact lies in the profit losses of infected herds. Mycoplasmas are the smallest free-living bacteria (between 0.3 and 0.8 μm in diameter) and are characterised by the lack of a cell wall. Clinical signs differ from one species to another: respiratory symptoms for Mycoplasma gallisepticum, infectious synovitis or subclinical infection of the upper respiratory tract for Mycoplasma synoviae, and specific immunosuppression in turkey for Mycoplasma meleagridis. Eggshell apex abnormalities are also induced by Mycoplasma synoviae.

An early detection of the infection of the flocks is a key point to putting in place corrective measures such as treatment, vaccination or disposal of the batch.

Mycoplasma gallisepticum can be diagnosed by different methods such as detection of specific antibodies with rapid plate agglutination, hemagglutination inhibition or enzyme linked immunosorbent assay (ELISA), by direct isolation or by detection of specific DNA by polymerase chain reaction (PCR). Culture of Mycoplasma gallisepticum is time-consuming. PCR, which is referred as a confirmation test (Asgharzade et al. 2013), can give negative results after an infection if an antibiotic treatment has been implemented during this period. Thus, the usual detection methods rely on both antibodies detection and PCR.

Rapid plate agglutination (RPA) tests are known to detect antibodies before ELISA as they detect IgM and IgY, whereas ELISA detects only IgY. In some studies, RPA shows a positive reactor 7 to 10 days after inoculation or vaccination (Asgharzade et al. 2013; Kleven 1975, 1998). In other studies (Pakpinyo et al. 2006) a positive result is only reported three weeks after inoculation, like ELISA.

The aim of this experiment is to assess the detection time of antibodies after MG experimental infection in chicken with these two methods.

Experimental Procedure

Animals: Ten 11-week-old Mycoplasma gallisepticum and Mycoplasma synoviae-free chickens were raised in individual cages. Daily check-ups were performed to avoid unnecessary suffering (1 chicken was euthanised within 2 days after challenge).

Strain and challenge: All the animals were challenged at Day 0 by intranasal route with 100 μL of a solution containing a Mycoplasma gallisepticum strain at a concentration of 1.10^9 CFU.ml^-1.

Serum collection: Chickens were bled aseptically from brachial vein, in sterile blood collection plain tubes, for serology, every 2 to 3 days, starting from the challenge day until the end of the experiment at day 25.

Tests: The serological tests were performed with Biovac’s RPA (Mycoplasma gallisepticum, Mycoplasma meleagridis, Mycoplasma synoviae, Salmonella Enteritidis, Salmonella Pullorum Gallinarum), Soleil’s RPA (Mycoplasma gallisepticum and Mycoplasma synoviae) and ELISA tests (Mycoplasma gallisepticum and Mycoplasma synoviae). All RPA tests were carried out in the Biovac laboratory. ELISA and other Biovac RPA tests were carried out in an RPA COFRAC-certified laboratory.

Dilution: RPA tests in the Biovac laboratory were done using pure and diluted sera. Dilution rates were 1/2, 1/4 and 1/5. In the COFRAC laboratory, RPA tests followed the French mandatory procedure in which sera are tested non-diluted and with 1/5 dilution rate and heating.

Heating: All the sera were heated at 56°C during the 30 minutes before being diluted. Pure sera were not heated.

Results

RPA with Biovac tests performed at Biovac: Figure 1 shows the evolution of positive RPA reactors observed from D0 (challenge day) to D25.

Agglutinations, when using pure sera, appeared at day 6 and shot up to 80% at day 8. Positive results with dilutions to 1/5 reached a first plateau at 10% starting at day 8 and a higher one (33%) at day 20. In comparison, ELISA’s positive results started at day 25.

RPA with Biovac tests performed by the external laboratory (Figure 2): The general pattern was rather similar with a quick
and strong reaction of the pure serum. Diluted sera curved on the lower end.

On closer inspection, the latter differed slightly with a first plateau at 20% of positive results and a peak at about 70% on day 22.

RPA with Soleil tests performed at Biovac (Figure 3) The product had a high sensitivity with a peak of 100% and 65% for ½ and ¼ dilutions respectively on day 20. The detection time was slightly increased compared to Biovac products but still one week before ELISA (from day 6 to day 18 for 1/5 dilution).

Statistical Interpretation
Considering the low number of animals challenged, the data did not follow a normal distribution. The Wilcoxon test was used to compare the mean day of detection of antibodies between each test with R data analysis software. There was a statistical difference between the two RPA tests using pure or ½ diluted sera, and ELISA (p<0.01).

At ¼ dilution, there was a statistical difference between Soleil test and ELISA (p=0.01) and at 1/5 dilution between Biovac test and ELISA (p<0.02).

Specificity (Table 1): The specificity of the RPA and ELISA products was assessed by testing the sera with Mycoplasma meleagris, Mycoplasma synoviae, Salmonella Enteritidis and Salmonella Gallinarum Pullorum.

<table>
<thead>
<tr>
<th>Test</th>
<th>Product</th>
<th>Operator</th>
<th>Number of positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA</td>
<td>Mycoplasma meleagris</td>
<td>Biovac</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Biovac synoviae</td>
<td>Biovac</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Biovac synoviae</td>
<td>External Laboratory</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mycoplasma synoviae</td>
<td>Soleil</td>
<td>3 (pure serum)</td>
</tr>
<tr>
<td></td>
<td>Salmonella Enteritidis</td>
<td>Biovac</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Salmonella Gallinarum</td>
<td>Biovac</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pulorum</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Salmonella Gallinarum</td>
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</tr>
<tr>
<td></td>
<td>Pullorum</td>
<td>Soleil</td>
<td>0</td>
</tr>
<tr>
<td>ELISA</td>
<td>Mycoplasma synoviae</td>
<td>External Laboratory</td>
<td>2</td>
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</tbody>
</table>

Table 1: Number of false positive results on tests other than Mycoplasma gallisepticum results throughout the entire experiment. Total number of tests: 972

Out of the 846 additional RPA tests performed, only one turned out to be positive on pure serum alone (Soleil range MS on one chicken on day 11). On the other hand, the ELISA tests displayed positive results twice on day 15 and 20 for the MS assay (126 tests in total).

Discussion
In this experiment, positive reactors in ELISA tests were detected 25 days after inoculation, which is slightly longer than in other experiments (Kleven 1998; Pakpinyo et al. 2006; Asgharzade et al. 2013). In the case of rapid plate agglutination, positive results with pure sera were observed less than a week after challenge and the 100% mark was reached within 11 to 18 days. Dilution to 1/2, 1/4 and 1/5 were performed to comply with all countries and/or laboratory regulations. As expected, the strength and the time to reaction decreased with the dilution ratio. It was however possible to observe a difference of at least 5 to 17 days between ELISA and RPA using 1/5 and pure dilutions respectively. These few days of difference can have important consequences in regularly tested flocks.

RPA results seemed to be quite erratic. Positive results decreased on days 20 and 22, just before positive reactors were observed with ELISA tests. A possible explanation relies on the presence of different types of antibodies produced by the immune system. IgM appears sooner compared to IgY and if ELISA only detects IgY, RPA reacts with both. The slight decrease described above could therefore be attributed to the transition between the two types of antibodies (point A on Figure 4).

RPA tests are prone to false positive reactions (Nassik et al. 2013). In this study, the sensitivity and specificity of all the tests were good. It should be noted that the chickens were not vaccinated before as it has been described that false positive results are associated with inactivated vaccines that contain oil emulsion or virus vaccines prepared in cell cultures supplemented with mammalian serum (Butcher 2012).
False positive results can also be associated with frozen sera which have been thawed before testing.

This is why before interpreting any erratic results, some points need to be carefully checked in the laboratory:

- Sera must be sampled at least 24 hours before testing, but not be more than 72 hours old;
- Positive and negative controls must be done before each series of tests;
- Antigens are gently but thoroughly and regularly shaken;
- The same volume of antigen and serum has to be used;
- All components are brought to room temperature, between 20 to 25 degrees Celsius, before use;
- Avoid any contact between sera and antigens before mixing;
- Shaking must last exactly 2 minutes for poultry sera and 3 minutes for turkey sera;
- Reading must be done within 30 seconds after shaking.

Conclusion

In this experiment, chickens challenged with MG produced immune response detected by RPA 5 to 17 days before ELISA. Positive reactors were observed 6, 15 and 17 days after inoculation with respectively 1/2, 1/4 and 1/5 diluted sera in the case of Soleil RPA tests and 25 days for ELISA. Biocat tests were positive 6 days after challenge with ½, ¼ and 1/5 dilutions.

Serological tests are performed for a screening of the flock’s status, not for a diagnosis (Feberwee et al. 2005). Considering that detection programmes generally rely on monthly sampling, a few days of difference in detection can be of immense importance for biosecurity. In France, it is compulsory to perform detection programmes with RPA. Thanks to this method, Mycoplasma gallisepticum has dramatically decreased in France during these last few years. The most important issue in the use of this quick and inexpensive method is to follow the instructions very strictly.

References


Caroline Pommellet is Director of the division of Reagents and Swine Autogenous Vaccines at Biocat Laboratories (France) since 2013. She works with the Research and Development department and is in charge of many trials in the field as well as in the animal house laboratory. Following her graduation from the Ecole nationale vétérinaire de Maisons-Alfort in 1985 she was a field practitioner in Brittany France, for 15 years. Since then she has worked with the agroindustry as General manager at Zootech laboratories, in the Even group (responsible for the medicated feed for the four feed plants) and the Merial group. She has been for several years a member of the French Scientific Committee for Feed industry. She has published numerous articles on her work in scientific journals.

Email: caroline.pommellet@biovac.fr