

qPCR kits for fecal samples to identify the shedders of Paratuberculosis or *Salmonella dublin* among highly ELISA-positive cows

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The use of ELISA tests in milk and blood is a common way to find cows infected with Paratuberculosis (MAP), and in Denmark, *Salmonella dublin* as well. Because of the need for repeated sampling for MAP (due to low specificity of the ELISA test) the cost is relatively high. The results are a decreasing participation in the Danish MAP control program.

Among herds ELISA-positive for *Salmonella* in bulk tank milk, it is common to look for highly ELISA-positive cows, but often many cows are antibody positive, even infection-free animals, which makes it difficult to locate cows shedding the *Salmonella*. Therefore, in both disease complexes, there is a need for a test that enables farmers to find the right cows to cull, in order to reduce the presence of the pathogens in the herds.

DNA Diagnostic A/S, Risskov, Denmark, has developed two new qPCR tests, 'ParaTB', and 'Salmonella 4 Cows', both of which can be coupled to the same fecal extraction protocol, also developed by DNA Diagnostic A/S.

For the fecal test for MAP, a total of 46 cows listed as high ELISA-positives and 58 control cows (low ELISA signal) in the Danish control program, from the same herds, were tested by the ParaTB qPCR test. Only 26 (57%) of the high ELISA-positive cows was found to be high shedders of MAP bacteria in the fecal samples (Ct values <33). Of the 58 control cows 5 (9%) were shedding high numbers of MAP bacteria. Also, 169 cows from an assumingly negative herd were tested and all tested negative. Finally, fecal samples from 5 cows with diarrhea and clinical signs of MAP were tested, of which all tested positive (Ct values from 21,4 to 32,6).

For the fecal test for *Salmonella dublin*, a total of 55 high *Salmonella* ELISA positive (OD>100) cows, were tested, and only 7 (13%) were positive shedders of *Salmonella* bacteria. Among 402 cows with lower ELISA-positive *Salmonella* antibodies measurements in milk or blood, two extra shedders were found. All shedders were culled immediately. During the six months after the last PCR positive cows were culled, all newly introduced heifers were checked by milk ELISA at first test day. None of the animals showed seroconversion. This indicates that new infections seem to have stopped. On this farm, this is the first period in two years, that newly introduced heifers have not seroconverted for *Salmonella dublin* in the ELISA test before first test day.

Abstract

The newly developed qPCR for MAP, 'ParaTB', and the qPCR for Salmonella (Salmonella spp. + Salmonella dublin), 'Salmonella 4 Cows', have shown to be highly effective in finding cows shedding the bacteria in fecal samples, and thereby motivating the farmers to effectively reduce the shedding of bacteria by culling shedding cows immediately.

Keywords: Paratuberculosis, Salmonella dublin, ELISA, qPCR, culling strategy

Introduction

The use of ELISA tests in milk and blood is a common way to find problem cows with Paratuberculosis (MAP), and in Denmark, Salmonella dublin as well. Because of the need for repeated sampling for MAP (due to low specificity of the ELISA test) the cost is relatively high. It is common knowledge among farmers, that even high antibody-positive cows for MAP, are not going to show symptoms right away. Saxmose & Kirkeby (2016) found that 29% of cows listed as highly positive cows in Denmark, calved after being listed as high ELISA positives and stayed in the farm for an average of 1.4 years (max. 6.9 years). The results are a decreasing participation in the Danish MAP control program.

Among herds positive for Salmonella in bulk tank milk, it is common to look for highly ELISA-positive cows, but often many cows are antibody positive, which makes it difficult to locate cows shedding the Salmonella. Therefore, in both disease complexes, fecal qPCR is a tool to find the right cows to cull, in order to reduce the presence of the pathogen in the herds.

Material and methods

MAP trial

Fecal samples were collected in nine Danish dairy herds, between October 2018 – April 2019. In total 46 cows listed as high MAP ELISA-positives in the Danish control program and 58 control cows (ELISA negatives) from the same herds, was tested by the ParaTB qPCR test, DNA Diagnostic A/S, Risskov, Denmark. In addition, 169 cows from an expected complete negative herd (found negative in the Danish control program), as well as 5 cows from three different herds, of animals showing clinical symptoms of MAP, such as reduced weight and diarrhea, were tested.

Salmonella trial

In total 55 cows with high Salmonella ELISA signal (OD>100), and 402 cows with lower ELISA-positive measurements in milk or blood, was tested by the Salmonella 4 Cows qPCR test, DNA Diagnostic A/S, Risskov, Denmark.

Sample treatment, DNA extraction and qPCR

Fecal samples were collected in 'Faeces Tube 76mm x 20 mm' (Sarstedt, O/N 80.734) using the accompanied lid scoop, and stored at 4 °C until DNA extraction, for a maximum of four days. DNA extractions were performed according to supplier protocol ('ParaTB' / 'Salmonella 4 Cows', DNA Diagnostic A/S). Briefly, 7 mL 'Buffer F' was added to the Sarstedt Feces Tube containing the feces samples, and mixed. The tubes were centrifuged at 1000x g for 1 minute and 250 µL supernatant transferred to the supplied 2 mL 96-deep-well plate containing 'Beads solution'. The deepwell plate was centrifuged at 5000x g for 5 minutes, supernatant removed and pellet washed with 1 mL 'Wash

Buffer', followed by a second centrifugation step. 120 µL 'Lysis-I mix' was added to the pellets, and samples homogenized. The samples were transferred to supplied 0.2mL tubes (8-well-strips), and incubated at 37°C for 20 min, 95°C for 15 min and 4°C for 5 min. The resulting lysate was centrifuged at 5000x *g* for 5 minutes, and 4 µL supernatant transferred to a well in either a 'ParaTB' qPCR plate or a 'Salmonella 4 Cows' qPCR plate, containing all necessary components for the qPCR reaction. qPCRs were performed on a CFX96 Touch (Bio-Rad), an ABI 7500 Fast (Thermo Fisher), or a MX3005p (Agilent). qPCR analysis according to supplier protocol ('ParaTB' / 'Salmonella 4 Cows', DNA Diagnostic A/S)

In table 1 it is shown that only 26 (57%) of the ELISA-positive cows was found to be high shedders of MAP bacteria in the fecal samples (Ct values <33). Of the 58 control cows, 5 (9%) were shedding high numbers of MAP bacteria. In the 169 fecal samples from cows in an assumingly negative herd all tested negative. Finally, fecal samples from the five cows with clinical signs of MAP all tested positive (Ct values from 21.4 to 32.6).

Table 2 shows that among the 55 high ELISA positive (OD>100) cows only 7 (13%) were positive shedding Salmonella bacteria.

Among the 402 cows with lower ELISA-positive measurements in milk or blood, two extra shedders were found. All shedders culled immediately after test results. None of the new introduced seronegative ELISA heifers showed seroconversion over a now 6-month period. This indicates that new infections seem to have stopped.

Results

Table 1. Fecal qPCR and ELISA results from 46 positive cows and 58 control cows in the Danish MAP control program. Also 169 cows from expected negative herds tested negative in both test (not shown).

| Fecal sample | ELISA in milk | |
|--------------|---------------|-----|
| | Pos | Neg |
| Pos <33 | 26 | 5 |
| Neg >33 | 20 | 53 |

Table 2. Fecal qPCR and ELISA milk and blod. Results from 55 high positive ELISA cows and 402 ELISA low positive or negative cows

| Fecal sample | ELISA in milk or blood | |
|--------------|------------------------|-----|
| | Pos | Neg |
| Pos < 37 | 7 | 2 |
| Neg >37 | 48 | 400 |

Conclusion

The newly developed qPCRs for MAP, ParaTB, and the qPCR for Salmonella dublin, 'Salmonella 4 Cows', have shown to be highly effective in finding cows shedding bacteria for these two infections in fecal samples, and thereby motivating the farmers to effectively reduce the shedding of bacteria by culling these cows immediately.

Follow up test in the Salmonella positive farm in the six months after the last PCR positive cows were culled, all newly introduced heifers were checked by milk ELISA at first test day. None of the animals showed seroconversion. This indicates that new infections seem to have stopped. For this farm, it is the first time in two years, that newly introduced heifers have not seroconverted for Salmonella (ELISA test) before the first test day.

List of references

Saxmose S. & C. Kirkeby, 2016. Dairy farmers' compliance with culling recommendation in paratuberculosis programme. <https://www.svepm.org.uk/posters.html>