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Parturition affects test-positivity in sheep with subclinical paratuberculosis; investigation following a preliminary analysis

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ABSTRACT

Within the context of an investigation focused at improving the effectiveness of test-and-removal for the control of ovine paratuberculosis, we recently conducted a preliminary study using ELISA and real time PCR to assess whether parturition affects test-positivity, in animals with subclinical paratuberculosis. Samples of faeces and blood were collected from 42 adult female animals, before (PP1) and after parturition (PP2), and before mating (PP3). In the preliminary stage of the analysis, only one of the animals tested reacted positively to ELISA (2.38%, 1 of 42), which corresponds to 2.8% of PCR-reactors (1 of 36). Therefore, the final stage the investigation was conducted using only real time PCR, which was applied to test samples of faeces collected from 85 animals, in 5 periods of sampling: 4–15/1–3 days (FP1/FP2) before and after parturition (FP3/FP4), and before mating (FP5). The result of the preliminary analysis indicated that PCR-positivity in terms of the number of shedders and the amount of MAP, is statistically significantly lower before parturition (PP1), whereas that of the final, higher in FP4 compared to FP5. Significantly higher levels of positivity in PP2 and FP4 were also recorded in connection with animals reacting positively to PCR more than once. In conclusion, in sheep with subclinical paratuberculosis, the period of 4–15 days postpartum is more suitable for the application of test-and-removal aiming to the control of the disease, using real time PCR. The use of ELISA for the same purpose is not recommended in the specific category of animals, due to low sensitivity.

1. Introduction

The *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a fastidious acid-fast pathogen that is ubiquitous in nature, and is classified as a member of the *Mycobacterium avium* complex (Inderlied et al., 1993; Harris and Barletta, 2001). MAP is recognized as the causal agent of a chronic intestinal degenerative disease that affects mainly ruminants, and is referred to as paratuberculosis or Johne's disease (Sweeney, 2011; Gautam et al., 2018; Marquetoux et al., 2018). The specific disease is considered a very potent threat to animal production, not only because of its negative impact on the productivity of the affected animals, but also because of its implication in a constantly increasing number of pathologic conditions of unknown etiology of humans (Behr and Collins, 2010; Naser et al., 2014; Waddell et al., 2015; Arru et al., 2016; Rathnaiah et al., 2017; Pierce, 2018). Though conclusive evidences regarding the association of MAP with the latter category of diseases are not available, the concern about human exposure to the specific pathogen through animal products increases dramatically the significance of the effective control of paratuberculosis of ruminants (EFSA AHAW Panel, 2017; Rathnaiah et al., 2017). In many cases however, especially in sheep, affected animals do not seroconvert before the final stage of the disease, which makes their detection difficult (Juste et al., 2005; Sohal et al., 2007; Bastida and Juste, 2011; Whittington et al., 2017). This is further complicated by the fact that these animals

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may not develop clinical disease with pronounced symptoms, for months (Whittington et al., 2017; Marquetoux et al., 2018). In consequence, and in the absence of a vaccine capable of preventing disease infection, the control of paratuberculosis becomes particularly challenging (Bastida and Juste, 2011; EFSA AHAW Panel, 2017; IAP, 2017; Barkema et al., 2018).

Within the context of an investigation that was focused at improving the effectiveness of test-and-removal for the control of ovine paratuberculosis in practice, we recently conducted a preliminary study using ELISA and real time PCR to assess whether parturition affects test-positivity, in animals with subclinical paratuberculosis (Mataragka et al., 2017). The preliminary stage of the analysis was conducted in a smaller animal population in order to assess more accurately the parameters that would be addressed in the final, such as sampling periods, methodology of sample collection in connection with parturition, and diagnostic approach. Based on the outcome of this investigation we proceeded to a more thorough analysis of the same subject using this time only real time PCR on a number of individuals and sampling periods that increased from 42 to 85 and from 3 to 5, respectively. The result of the latter study is assessed in combination with that of the preliminary, to deduct a conclusion regarding the time period during which, test-and-removal of MAP reactors in a sheep flock with subclinical paratuberculosis would be more effective.

2. Material and methods

2.1. Sample collection

The overall sampling plan including the characteristics of the targeted group of animals, the stages of the analysis (P: preliminary, F: final), the periods of sampling (P1-5), and the number of animals that were tested, is presented in Table 1. The animals tested in both stages of the analysis belong to the same flock (Mataragka et al., 2017), which consists of non-vaccinated animals with no cases of clinical paratuberculosis, but with a long record of positivity to MAP, based on faecal real time PCR and culture (Liandris et al., 2009; Taka et al., 2013).

For the selection of the animals to be tested in connection with the periods PP1/FP1 (1–3 weeks/4–15 days before parturition), no reversion to estrus and growth of the udder were used as indicators of pregnancy. During the specific periods (PP1/FP1), samples were collected from all the animals that could be identified as pregnant, based on these evidences. With regards to the period FP2 (1–3 days before parturition), samples of faeces were collected from the targeted population every 3 days before the expected date of partum, for approximately 15 days, and the analysis was conducted on the sample that was collected last, before parturition.

In both stages of the study, samples of rectal faeces (15 g) were collected from every animal, and were processed for DNA isolation and detection of MAP DNA using real time PCR. Additionally, samples of whole blood (5 ml) were processed for the detection of MAP-specific antibody with ELISA, which was conducted only in the preliminary stage of the analysis. Hence 626 samples were collected in total, 240 during the preliminary (120 faeces, 120 blood), and 386 during the final stage.

2.2. Sample analysis

Sample analysis was conducted as previously described (Mataragka et al., 2017).

In brief, detection of MAP specific antibody was performed in whole blood (n = 120), using a commercially available ELISA kit (IDEXX® Paratuberculosis Screening Ab Test, IDEXX Laboratories Inc., U.S.), according to the instructions provided by the manufacturer.

The Nucleospin® Tissue kit (Macheray-Nagel GmbH & Co. KG, Germany) was used for DNA isolation from samples of faeces (n = 506), after homogenization in double distilled sterile water and processing of the supernatant in a Mixer Mill (Retsch, Germany) with 2:1 v/v glass beads (Adiagene, France). Real time PCR was performed using the primers F: 5′-AATGACGGTTACG GAGGTGGT-3′, R: 5-GCAGTAATGGTCGGCCTTACC-3′, and the probe, P: 5′ FAM-TCCACGCCCGCCCAGACAGG-TAMRA 3′, to amplify a 76 base pair fragment within the *IS900* element of MAP (Kim et al., 2002, 2004; Liandris et al., 2014; Mataragka et al., 2017).

Sample testing was conducted in compliance with ISO17025 requirements (ISO/IEC 17025:2017) in connection with ELISA, DNA isolation, and real time PCR. The analysis incorporated the recommended measures of quality assurance, including use of positive and negative controls, validation of the DNA quality, detection of PCR inhibitors, and confirmation of the specificity of the amplification product using sequence analysis (Mataragka et al., 2017).

2.3. Statistical analysis

The qualitative real time PCR and ELISA results are presented as frequencies (percentages) and were compared among the sampling periods with the non-parametric Cochran's Q test, which is used for comparing more than two related/paired proportions. Pair wise comparisons were done with the non-parametric McNemar's test. Depending on the case, comparison between independent proportions was conducted using the chi-squared test, and the two, or the one-tailed z-tests. Based on the outcome of the Shapiro-Wilks test, the quantitative real time PCR results (amount of MAP DNA detected in samples of faces) do not follow a normal distribution, and are therefore presented as medians (lower quartile, upper quartile); comparison of the respective counts between the breading stages was conducted with the non-parametric Friedman test. Post-hoc comparisons were done with the non-parametric Wilcoxon Singed-Rank test. Statistical analysis was performed

Table 1
The sampling plan consisting of the stages of the analysis (P: preliminary, F: final), the periods of sampling (P1-5), and the total number of animals that were tested.

Preliminary analysis: October 2014 - June 2015					
Sampling period	PP1		PP2	PP3	
Sampling stage conducted	October - November 2014 1–3 weeks before parturition		December 2014 - January 2015 2–15 days after parturition	May - June 2015 Before following mating season	
Number of animals	36		42	42	
Final analysis: December 2016 - July 2017					
Sampling period	FP1	FP2	FP3	FP4	FP5
Sampling stage conducted	December 2016 4–15 days before parturition	December 2016 1–3 days before parturition	December 2016 - January 2017 0–3 days after parturition	January 2017 4–15 days after parturition	June - July 2017 Before following mating season
Number of animals	60	81	85	85	75

using the statistical software SPSS (version 16.0, SPSS Inc., Chicago, IL, U.S.A); the significance level was set for all tests at 5%.

3. Results

The results recorded in each of the stages of the analysis and the periods of sample collection are presented in Table 2, together with those of the respective statistical analysis.

The proportion of the animals that reacted positively to real time PCR was statistically significantly different between the periods of sample collection of the preliminary (Cochran's Q test Q = 13.4, P = 0.0012) and the final (Cochran's Q test Q = 9.89, P = 0.042) stage of the analysis. With regards to the preliminary study, the proportion of PCR-reactors in PP1 (before parturition) was significantly lower, compared to those of PP2 (after parturition), (McNemar's test X^2 = 10.67, P = 0.0015), and PP3 (before mating), (McNemar's test X^2 = 7.12, P = 0.0127). In the final stage of the analysis, the proportion of positive animals of FP4 (4–15 days after parturition) was significantly higher compared to that of FP5 (before the mating season), (McNemar's test X^2 = 7.53, P = 0.006), whereas the difference of the proportion of positive animals in all other stages of sampling was not statistically significant (McNemar's tests P > 0.05).

Comparing the level of positivity recorded in the two stages of the analysis indicates statistically significant difference in all the respective periods of sampling i.e. before parturition (PP1: 13.9 ± 11.30%, FP1: 38.3 ± 12.30%, two-tailed z-test Z = 2.55, P = 0.0108), after parturition (PP2: 59.5 ± 14.85%, FP4: 40.0 ± 10.41%, two-tailed z-test Z = 2.07, P = 0.0385), and before the mating season (PP3: 47.6 ± 15.10%, FP5: 21.3 ± 9.27%, two-tailed z-test Z = 2.96, P = 0.0031). The relevant percentages were in all cases higher in the preliminary stage of the analysis compared to the final, except for those corresponding to the period before parturition (FP1 > PP1).

The quantitative analysis of MAP DNA that was detected in the samples of faeces using real time PCR indicates a trend, which is consistent with that of the qualitative analysis with regards to each of the two stages of the study (Table 2). In more detail, the median values of the score numbers of the real time PCR counts between the breading stages differed statistically significantly in the preliminary stage of the analysis (Friedman test X^2 = 15.09, P = 0.0005), with that corresponding to the period before parturition (PP1) being significantly lower, compared to those postpartum (PP2), (Wilcoxon Singed-Rank test Z = 3.30, P = 0.0010), and before mating (PP3), (Wilcoxon Singed-Rank test Z = 2.49, P = 0.0129). The counts were also statistically significantly different among the 5 periods of sampling with regards to the final stage of the analysis (Friedman test X^2 = 13.12, P = 0.0107), with the respective median value of the score numbers of the real time PCR counts corresponding to FP4 (4–15 days postpartum) being significantly higher than that of FP5 (before mating), (Wilcoxon Singed-Rank test Z = 2.52, P = 0.0117).

Only one of the tested animals reacted positively to real time PCR in all periods of sample collection (FP1-5), which corresponds to 1.4% of the real time PCR reactors (1 of 70). In the preliminary stage of the analysis, the proportion of the animals that reacted positively to real time PCR in one, and in two, of the three periods of sampling was 61.1% (22 of 36) and 38.9% (14 of 36), respectively; the difference between the proportions was statistically significant (one-tailed z-test, Z = 1.88, P = 0.0298). In the final stage, the proportion of the animals that reacted positively to real time PCR in one, two, three and four periods of sampling was 47.1% (33 of 70), 27.1% (19 of 70), 15.7% (11 of 70), and 8.6% (6 of 70) of the positive reactors respectively, and the difference between the proportions was also statistically significant (chi-square test X^2 = 35.71, P < 0.0001).

In connection with the preliminary stage of the analysis and the animals reacting positively in at least two of the sampling periods (n = 14), those that produced the comparatively stronger PCR result (lower Ct value) in PP1, PP2, and PP3 were 7.1% (1 of 14), 64.3% (9 of 14), and 25.6% (4 of 14), respectively. These proportions are statistically significantly different (Cochran's Q test Q = 6.50, P = 0.0388), and the percentage that corresponds to PP2 is significantly higher compared to that of PP1 (McNemar's test X^2 = 5.44, P = 0.0126). The relevant results recorded in the final stage of the analysis with regards to the animals reacting positively to real time PCR in at least two of the sampling periods (n = 37), were 16.2% (6 of 37), 10.8% (4 of 37), 27.0% (10 of 37), 32.4% (12 of 37), 13.5% (5 of 37), in connection with in FP1, FP2, FP3, FP4, and FP5, respectively; the difference between the proportions is also statistically significant (Cochran's Q test Q = 13.62, P = 0.0086), and the percentage that corresponds to FP4 (4–15 days postpartum) is significantly higher compared to that of FP5 (McNemar's test X^2 = 6.23, P = 0.0126).

The analysis conducted using ELISA was positive in connection only with a 3-year old animal that reacted positively in all stages of sample collection.

4. Discussion

The result of the preliminary stage of the analysis indicated that in a flock of sheep with subclinical paratuberculosis, the percentage of animals that react positively to the detection of DNA belonging to MAP using real time PCR applied in samples of faeces is statistically significantly lower in the period of 1–3 weeks before parturition (PP1), compared to those after parturition (PP2), and before mating (PP3), (Mataragka et al., 2017). The outcome of the final stage of this study indicates a tendency of increase in the level of positivity, from FP1 to FP4, which peaked in the latter period of sampling (FP4), reaching its maximum value that was statistically significantly higher compared to that before mating (FP5). The respective quantitative results are consistent with those reported above in connection with each of the two stages of the analysis. In more detail, the median value of PCR score numbers is statistically significantly lower in PP1 compared to PP2 and PP3, and higher in FP4 compared to FP5 (Table 2).

The fact that the outcome of the preliminary and the final stage of the analysis is similar but not identical is probably associated with sample size, which in the former was approximately half of that of the latter (n = 42 in P, n = 85 in F). This probably accounts

Table 2
The result recorded in samples of faeces tested for the detection of MAP DNA using real time PCR, and that of the relevant statistical analysis. Qualitative (positivity %) and quantitative (real time PCR counts) results are presented as frequencies (percentages) and medians (lower quartiles, upper quartiles), respectively. Superscript letters "a" and "b" are used to denote statistically significant difference (P ≤ 0.05): frequencies and medians with different superscript letters are statistically significantly different, as opposed to those with the same.

	Preliminary analysis			Final analysis				
	PP1	PP2	PP3	FP1	FP2	FP3	FP4	FP5
No animals /No of positive	36 / 5	42 /25	42 /20	60 /23	81 /31	85 /29	85 /34	75 /16
Positivity (%)	13.9 ± 11.30^{a}	59.5 ± 14.85^{b}	47.6 ± 15.10^{b}	38.3 ± 12.30^{ab}	38.3 ± 10.59^{ab}	34.1 ± 10.08^{ab}	40.0 ± 10.41^{b}	21.3 ± 9.27^{a}
Real time PCR counts	0 (0, 0)^{a}	0.01 (0, 0.01)^{b}	0 (0, 0.01)^{b}	0 (0, 0.01)^{ab}	0 (0, 0.01)^{ab}	0 (0, 0.01)^{ab}	0 (0, 0.01)^{b}	0 (0, 0)^{a}

also for the smaller variation in the level of positivity recorded in the periods around parturition in the final stage of the analysis (FP1-4) compared to the preliminary (PP1 and 2). However, in spite the inconsistency of the results at the level of statistical significance, one is compelled to note that the variation in the proportion of PCR reactors between the periods of sampling is similar in both stages of the analysis, and indicates higher counts after parturition i.e. in PP2 and FP4 (PP1 = 13.88%, PP2 = 59.52%, PP3 = 47.6%; FP1 = 38.33%, FP2 = 38.27%, FP3 = 34.11%, FP4 = 40%, FP5 = 21.3%). Notably, a single real time PCR test applied in a sample of faeces in the specific periods (PP2 and FP4) allowed detection of 59.5 ± 14.85% and 40.0 ± 10.41% of the reactors, respectively (Mataragka et al., 2017). This pattern is supported by the findings that were notably recorded at a statistically significant level in both stages of the analysis with regards to single, and multi-reactors, i.e. animals reacting positively to real time PCR once or more, respectively. In more detail, the percentage of single-reactors for which the positive result was recorded after parturition (PP2 and FP4) was higher in both stages of sampling compared to the other periods, as was the percentage of multi-reactors that produced the comparatively stronger real time PCR-positive result.

The variation in faecal shedding of MAP associated with parturition is addressed to the best of our knowledge for the first time through this study, with regards to sheep. Relevant studies that are thoroughly reviewed in our preliminary report (Mataragka et al., 2017), have been conducted in cattle and sheep in connection with parturition and stress, respectively, and provide conflicting results. The latter is perhaps not surprising given the number of factors that influence the outcome of the specific investigation, such as the efficiency of the diagnostic tests used (low limit of detection, sensitivity, specificity, false reactions), exposure of the animals to stress not associated with the study, nutrition, health status, and genetic predisposition of the animals towards a predominantly Th1 or Th2 immune response (Marquetoux et al., 2018), contact with other animals or exposure to environmental sources of infection, age, etc. In order to decrease the bias generated by some of these parameters, this assessment relied on ISO17025 accredited diagnostic tests, it covered a period of approximately three years, and was conducted in animals maintained under a continuously monitored diet, with no exposure to pasture or other animals.

Regarding the structure of the experimental design, it should also be noted that the preliminary investigation relied on ELISA and real time PCR because, on the contrary to *in vitro* isolation of MAP that is time consuming (Harris and Barletta, 2001; Collins, 2011), the specific tests are rapid and can support high throughput analysis (OIE, 2014; IAP, 2017). Furthermore, using real time PCR to investigate the period of breeding during which the number of MAP shedders is maximized, could also prove significant in improving the sensitivity of the *in vitro* isolation of the specific pathogen. Given that only one of the animals tested reacted positively to ELISA (2.38%, 1 of 42), which corresponds to 2.8% of the PCR-reactors (1 of 36), the final stage of the analysis was conducted using only real time PCR. In this regard it is perhaps worth noting that the relative inefficiency of ELISA compared to real time PCR constitutes unfortunately one more obstacle in applying an effective test-and-removal plan for the control of ovine paratuberculosis. The complexity of the latter becomes probably more apparent by the fact that, in spite the low minimum detection limit of real time PCR (Kim et al., 2002, 2004), the intermittent shedding of MAP in faeces caused considerable variation in the level of positivity in the tested animals over time. Interestingly, the decrease in the number of animals reacting positively to real time PCR in more than one periods of sampling, was statistically significant in the preliminary stage of the analysis ($P = 0.0298$), and dramatic in the final ($P < 0.0001$). It is perhaps interesting to note with regards to the real time PCR-reactors that none of the animals tested reacted positively in all stages of sampling (0 of 86), whereas only 10.47% (9 of 86) of them reacted negatively, which shows rather clearly how difficult it is to detect MAP-reactors in practice, even in a flock of sheep consisting almost exclusively of infected animals.

Admittedly, the structure of this investigation is not appropriate in terms of conducting an etiological analysis of its outcome. However, analysing the results that were recorded in the preliminary investigation we speculated that the lower level of test-positivity before parturition could be associated with the concurrent decrease in gastric activity (Mataragka et al., 2017). This issue was investigated more thoroughly in the final part of the study, in which sample collection was conducted in two stages before (1–3/ 4–15 days), and after parturition. The results that were recorded in FP2 and 3 correlated to those of the periods corresponding to 4–15 days before, (FP1) and 4–15 days after parturition (FP4), indicate that faecal shedding of MAP remains relatively low throughout FP1 to FP3, and it is increased in FP4, which is consistent with the potential association between decreased gastric activity due to parturition and lower percentage of MAP shedders. Notably the specific factor, i.e. the decreased gastric activity, probably accounts for the fact that the proportion of positivity around parturition (FP1-4) varied, but not at a statistically significant level. This however does not explain the statistically significantly higher level of MAP shedders 4–15 days postpartum (FP4), compared to (FP5), since if the only factor that determined the variation in the excretion of MAP were parturition-associated decreased gastric activity, the proportion of reactors in FP4 would not be higher than that of FP5, certainly, not at a statistically significant level. In this respect, it can be speculated that though the small variation in the percentage of MAP-shedders in the period around parturition could be associated with decreased gastric activity, the increased positivity in terms of the number of reactors and the amount of MAP DNA, 4–15 days postpartum compared to the period before mating (dry season), is probably associated with the onset of lactation. The causal factor of the latter could be stress or the concurrent increase in gastric activity, which later on returns to normal, leading to lower levels of positivity (number of MAP-shedders and amount of MAP DNA) in FP5. Regardless causation, it is noteworthy that increased faecal shedding of MAP after partum constitutes a factor which would maximise disease transmission, since animal exposure occurs when MAP shedding and host sensitivity are at their highest level.

In conclusion, in sheep with subclinical paratuberculosis, the proportion of animals that react positively to real time PCR applied in samples of faeces for the detection of DNA belonging to MAP, as well as that of the animals reacting strongly positively to the specific test, are statistically significantly higher in the period of 4–15 days postpartum, compared to that before mating. In this respect, the specific period is more suitable for the application of test-and-removal aiming to the control of paratuberculosis, using real time PCR. The use of ELISA for the same purpose is not recommended in connection with the specific category of animals, due to low sensitivity.

5. Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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