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Mycobacterium avium paratuberculosis seroconversion in dairy cattle and its association with raised somatic cell count

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ABSTRACT

This retrospective case-control study investigates the relationship between seroconversion to *Mycobacterium avium paratuberculosis* (MAP) and raised somatic cell count. The study consists of 112 case cows from 3 dairy farms in the UK, for each case cow with a positive antibody titre, there was a seronegative control cow for comparison. Seroconversion was monitored using milk ELISA antibody titres for MAP taken at quarterly intervals. Somatic cell counts (SCC) were recorded at the time a positive antibody titre was first recorded as well as at the previous and subsequent milk recording in order to explore a temporal relationship between the two events. The previous and subsequent milk recordings were a month before and after seroconversion was identified.

The results showed that cows that were infected with MAP had an increased SCC around the time that they first became seropositive providing evidence for a temporal relationship between the two events; high SCC were particularly prevalent before and at the time of first

detecting seroconversion. The explanation is being discussed that potentially an underlying, currently not studied, factor may be predisposing both events, the progression of paratuberculosis is predisposing the host to mastitis, or indeed intramammary infections help initiate paratuberculosis progression.

Keywords

paratuberculosis, subclinical mastitis, dairy cow, seroconversion

INTRODUCTION

Paratuberculosis, also known as Johne's disease, is a chronic, progressive, enteric disease caused by *Mycobacterium avium paratuberculosis* (MAP), which primarily affects ruminants. In addition to the impact of clinical Johne's disease on cows, there is an interest in the disease due to the reported association between MAP and the enteric condition Crohn's disease in humans[1], which has driven efforts to improve disease detection and its subsequent control.

When the animal is exposed to the pathogen most studies agree that the main portal of entry is the ileum where the MAP organisms are taken up by M cells within the Peyer's patches[2,3]. MAP organisms can be present in submucosal macrophages 5 hours after inoculation of a calf's ileum[2], and due to MAP's ability to survive within macrophages it is likely that the organisms will persist within these cells[4,5]. At the early stage of the disease, the host's immune defences prevent any outward clinical signs and contain the pathogen within the intestine and its associated lymphoid tissue through granuloma formation. As the disease progresses though this granulomatous response becomes more severe and diffuse, eventually becoming the cause of the clinical signs[6].

48 An important stage of the infection's progression is the transition in immune response from
49 one which is predominantly cell-mediated, stimulated by Th-1 cells and aimed against
50 intracellular pathogens; to one which is antibody-mediated, stimulated by Th-2 cells and
51 aimed against extracellular pathogens[7]. In the early stages of infection there is a strong bias
52 towards a Th-1 immune mediated response; the cytokine interferon-γ has a crucial role in
53 this bias[8,9,10] and is found in higher amounts from animals infected with MAP along with
54 other proinflammatory cytokines inducing a strong cell mediated immune response[11,12].
55 With clinical progression, a switch from a mainly cell mediated response to a humoral
56 immune response occurs[12,13,14]. This event coincides with an increase in faecal shedding of
57 MAP, and seroconversion is therefore used to indicate MAP shedding cows in the control of
58 paratuberculosis[7,15,16].
59 An impaired immune system due to the infection with MAP is hypothesised to be the reason
60 that these animals are more prone to subclinical mastitis [17,18]. Although some studies have
61 found no significant difference in somatic cell count (SCC) levels in the milk between
62 paratuberculosis affected and non-affected animals[19,20,21,22,23], others reported an increase
63 in SCC[24] as well as an increase in culling rates due to mastitis associated with MAP
64 infection[25]. A longitudinal study reported a first high SCC in 46% of the cows before MAP
65 antibodies were found and reversely 40% of the cows were identified as MAP positive first
66 [26]. It is, however, uncertain what the direction is of causality of this association. In order to
67 gain a level of insight into this, in this study we have looked into the temporal relationship
68 between MAP infected cattle and subclinical mastitis. This looks specifically at the moment
69 a first seropositive result is recorded, using this as an indicator for the disease's transition in
70 immune response [7,27] and somatic cell count is used as an indicator of intramammary

infection[28]. We examine the SCC before, after and at seroconversion to MAP in three UK dairy herds, in a retrospective longitudinal matched case-control study.

MATERIALS AND METHODS

Data Sources

The sample population consisted of all milking cows from three Holstein-Friesian dairy farms in England. The farms were selected based on their long-term testing for paratuberculosis and were taking part in the Herdwise Johne's Screening Programme (National Milk Laboratories, Chippenham, UK). Milk samples were taken on a monthly basis for National Milk Records to measure SCC. These milk samples were additionally tested every three months for antibodies against MAP using an ELISA. Antibody titres, SCC levels cow identity, and recording dates were obtained from National Milk Records using 'Herd Companion'. Data was taken from Farm 1 between May 2009 and May 2013; Farm 2 between March 2010 and June 2013; and Farm 3 between May 2011 and August 2013. Any data from the 6-week period after a tuberculin testing was excluded. None of the cows in this study have had a MAP vaccine administered.

Definitions

Paratuberculosis disease status

The date of first seropositive antibody response was defined as Time0. Seropositive as defined by the laboratory at >30 S/P % using the ELISA antibody test. All recruited seropositive cows had been seronegative on at least two tests prior to seroconversion. Dependent on subsequent antibody responses, positive cows were then grouped into antibody response groups (ARG). The groups consist of:

- High – a seropositive occurs with all subsequent tests are positive (minimum of two).

- Progressive – one or more positives occur, followed by one or more negative results, before another positive occurs with all subsequent tests to this second positive being positive (minimum of two).
- Transient – one isolated positive occurs with all subsequent tests seronegative (minimum of two).

Cows that were seropositive but could not be grouped due to missing data were excluded from the study.

Within each farm, one control cow was matched with each positive cow based on same parity and calving dates within one month of each other. The control cow would have a negative antibody result for MAP which corresponded to the first positive antibody result of the case cow; the same day that these antibody results were recorded was known as 'Time0'.The matched controls were seronegative throughout the testing period and in case there were multiple control cows available, one was selected randomly.

Somatic Cell Count

The point that a positive antibody result was first recorded was defined as Time0, and milk recordings before, at and after Time0 were used to evaluate the occurrence of subclinical mastitis. As dry periods and missed milk recording may skew the data, milk recordings more than 100 days apart from Time0 were excluded from the dataset. Due to the range and non-normal distribution of SCC, SCC was converted using the natural logarithm (Ln(SCC)). The Ln(SCC) values produced a bell-shaped histogram and had non-significant Kolmogorov-Smirnov values; Ln(SCC) values were therefore considered to have a normal distribution. We reported the summarised SCC levels as continuous SCC ($x10^3$cells/ml) by converting the LnSCC back to SCC. In addition to the continuous data, LnSCC, and as proxy for a (subclinical)

mastitis event SCCs were categorised into high and low using the cut off value of $200x10^3$cells/ml[29].

Statistical Analysis

All data was entered in a spreadsheet using Excel ® (Microsoft Inc.) and all statistical tests were run using SPSS 25® (IBM Inc.). The Generalised Linear Mixed Model function was used with Cow ID, nested in Case-Control Pair ID, nested in Farm ID as random effects. This is done to accommodate for the repeated measurements (Prior, Time0 and Post) and the Case-Control matching of the regression. The fixed effected were combinations of case vs. controls and specific ARGs at varying time points, to test an association with the dependent variables Ln(SCC) and recording of a high cell count event ($200x10^3$cells/ml). For the single time point analysis, paired T-tests (cases vs. controls) were used for the continuous data and conditional logistic regression for the binary data.

Ethical approval

Informed consent was obtained from each farmer contributing to the study. The Social Science Research and Ethical Review Board (SSRERB) of the Royal Veterinary College, University of London has examined and approved the research protocol (SR2017-1378).

RESULTS

Of the 1590 dairy cows sampled 374 had been seropositive to MAP at some point in the study period, 262 cows were excluded from further analysis due to not matching the inclusion criteria. The total number of cows with a positive antibody response (case cows) included in the study was 112, with 44 classified as high, 15 as progressive, and 53 as transient. Days in milk and parity were not different, p=0.765 and p=0.931 respectively, between the positive and negative cows, suggesting the matching on both parameters was

142 successful. Other than the somatic cell count reading as reported below, none of the
143 production parameters was significantly different between the positive cows and their
144 controls. This included milk yield (kg/day), fat and protein percentage and yields (kg/day). In
145 addition, no difference in either of these parameters was detected in the ARG subgroups.
146 Figure 1 shows the individual SCCs of the cows before, at (Time0) and after seroconversion.
147 The average SCC for all 224 cows within the study at the previous milk recording was 90
148 $x10^3$cells/ml (range: 6 to 3,789 $x10^3$cells/ml); at Time0 was 109 $x10^3$cells/ml (range: 2 to
149 4,185 $x10^3$cells/ml); and at the subsequent milk recording was 87 $x10^3$cells/ml (range: 6 to
150 4,603 $x10^3$cells/ml). In the 112 control cows, the average SCC at the previous milk recording
151 was 71.9 $x10^3$cells/ml, at Time0 was 84.0 $x10^3$cells/ml, and at the subsequent milk recording
152 80.1 $x10^3$cells/ml. Whilst in the case cows the median SCC at the previous milk recording
153 was 111.8$x10^3$cells/ml, at Time0 was 140.9 $x10^3$cells/ml, and at the subsequent milk
154 recording was 94.1 $x10^3$cells/ml.
155 The paired t-test showed a significant difference between the Ln(SCC) in case and control
156 cows at the previous milk recording ($p=0.003$) and at Time0 ($p<0.001$). There was no
157 significant difference found at the subsequent milk recording ($p=0.293$). SCC as a continuous
158 variable was then analysed within each ARG; distributions are shown in Table 1. The
159 statistical analysis showed that there was a significant difference between Ln(SCC) in case
160 and control cows at the previous milk recording and at Time0 for the High ARG ($p=0.010$,
161 $p=0.002$ respectively), but not for the subsequent recording ($p=0.058$).
162 Over time, the SCC values from before, at Time0, and after were not significantly different
163 for case cows ($p=0.058$) nor for control cows ($p=0.619$). Only for the control cows to the
164 transient ARG showed a significantly different Ln(SCC) over time ($p=0.029$), none of the

other ARG or their matched controlled showed significantly different Ln(SCC) levels over time.

At the 200 x10^3cells/ml threshold, 28.6% (64/224) of the studied cows had a high SCC at the previous milk recording, 33.0% (74/224) of them had high SCC at Time0 and 24.6% (55/224) of them had high SCC at the subsequent milk recording. At the milk recording previous to Time0, 36. 6% (41/112) showed high SCC in case cows compared to 20.5% (23/112) of control cows (Adjusted OR = 2.3 (95% CI: 1.2-4.2), p=0.009). High SCC were recorded 41.1% (46/112) of case cows at Time0 compared to 25.0% (28/112) of control cows (Adjusted OR = 2.0 (95% CI: 1.1-3.5), p=0.026), and 26.8% (30/112) of case cows had a high SCC at the subsequent milk recording compared to 22.3% (25/112) of control cows (Adjusted OR = 1.3 (95% CI: 0.7-2.4), p=0.450). There was no significant difference over time on the occurrence of high SCCs differed in case cows (p=0.061) and control cows (p=0.657).

Table 2 shows the occurrence of high SCC in each ARG; cows in the High ARG had more frequent high SCC at the previous milk recording (OR = 4.7 (95% CI: 1.5-14.7), p=0.008), at Time0 (OR = 7.8 (95% CI: 2.1-29.8), p=0.003) and subsequent recording (OR = 3.3 (95% CI: 1.1-10.3), p=0.037), based on the conditional logistic regression. In the Transient ARG, the frequency of high SCC recordings at the different time points was different in case (p=0.014) as well as control cows (p=0.034), but there was no significant difference observed over time in the other ARGs.

DISCUSSION

The results show that cases cows tended to have increased SCCs around the time that seroconversion occurred compared to matched control cows; both at the point the antibody test first became seropositive (Time0) as well as the milk recording approximately one

189 month earlier. In particular, this was the case in cows that had a consistently high antibody
190 response (High ARG). SCCs for case and control cows at the subsequent milk recording were
191 not significantly different. Cows that showed a progressively positive antibody response
192 (Progressive ARG) did not show a consistent pattern, which is maybe due to the limited
193 sample size (15 case cows).
194 The results suggest that cows that are progressing to MAP seropositive status have
195 concurrent elevated SCC as well as during the time period leading up to this point,
196 compared to matched control cows. A longitudinal study identified that a cow's age at the
197 point it had its first high SCC was positively associated with its age when it had its first
198 positive antibody titre to MAP[26]. This study however did not identify the sequence of
199 events, where we identified the occurrence of high SCC to precede the seroconversion. Part
200 of the complexity is the poor sensitivity of the serological test for Johne's disease, which
201 increases by repeated testing and also with age[30]. This could be explained via different
202 pathways; one explanation would be the presence of a confounding, underlying, and yet
203 unknown factor that predisposes cows to have a high SCC and to go through
204 paratuberculosis disease progression. Glucocorticoids are believed to influence
205 differentiated T-helper cells to alter their cytokine repertoire from a Th-1 to a Th-2
206 pattern[31]. Therefore, if the cow was put under significant stress and cortisol was released
207 into the circulation, the bovine immune response might become Th-2 dominated with both
208 conditions being affected similarly. A similar response is also seen around the time of
209 parturition and is believed to be one of the factors that contributes to increased incidence
210 of severe clinical mastitis during this period[32,33,34] as well as milk seropositive
211 paratuberculosis cases post-partum[35].

212 Another pathway to consider is that cows that have an escalating MAP infection predisposes
213 them to having a high SCC, a compromised immune system due to a MAP infection might
214 make the host's immune system susceptible to mastitis. Dotta and others showed that
215 subclinical paratuberculosis resulted in the reduction of migratory responses of
216 polymorphonuclear cells of the bovine immune system in vitro[36]. It has also been well
217 documented that the innate immune response is vital for cows to undergo spontaneous
218 cures and therefore a disease that prevents a rapid milk neutrophil response might
219 predispose cows to developing mastitis [34,37]. A review by Burton and Erskine (2003)
220 concluded the Th-1 immune response can be particularly beneficial against mastitis with the
221 immunoglobulin isotype IgG2 having a major role in this preferential cell mediated immune
222 response[34]. IgG2 secretion by B-cells is enhanced by interferon-γ and is the main opsonin
223 supporting neutrophil phagocytosis in milk[34,38]. If MAP infected macrophages are able to
224 subvert host immune responses to a Th-2 immune response as described above, the
225 question is whether MAP infection could negatively affect the host's ability to fight new
226 mastitis infections. Further research is needed to identify whether the lowered local
227 immune response (udder and intestine) are two separate events, or centrally linked.
228 The observation that the number of case cows that had a high SCC prior to (36.6%) and at
229 Time0 (41.1%), compared to after (29.5%) supports the notion of the third explanation:
230 having subclinical mastitis may accelerate cows' paratuberculosis disease progression.
231 Although mastitis infections are known to cause significant increases in cytokines that
232 promote a Th-2 immune response, these are generally produced alongside large quantities
233 of pro-inflammatory cytokines and interferon –γ which are central to the innate immune
234 response – the primary host determinant for dictating the outcome of the mastitis
235 infection[34,39,40]. In some cases of mastitis, somatic cell counts can increase to over

236 1,000x10^3cells/ml and with a milk yield of 25 litre per day, this is nearing 10% of the cows
237 granulocyte daily turnover, putting pressure on the cow's immune system and potentially
238 hindering its attempts to control a MAP infection[41].
239 Limitations to the study are the frequency of testing (SCC and milk ELISA), the limited
240 sensitivity of the test available and the lack of a measure of a central versus a local immune
241 response. Also, the study design does not allow us to be conclusive on what explanation is
242 best, the current data suggests that cows infected with MAP that were becoming
243 seropositive to the condition for the first time have increased somatic cell counts around
244 the same time. This association was evident at the first seropositive test, as well as at the
245 milk recording before, approximately a month earlier. This highlights a possible relationship
246 between the two conditions occurring. Further work involving more frequent testing is
247 required to determine a more precise temporal relationship than this study could achieve
248 with a comparison on quarterly testing for MAP and monthly testing of SCC. It remains
249 uncertain when cows will seroconvert, leaving a practical study design challenging to
250 execute. While treatments for mastitis were not included in this study, this could be
251 included to ensure the similar SCC in all groups at milk recordings subsequent to Time0 was
252 not a treatment effect.
253 Further work could look at the use of raised SCC as a potential means of selecting cows for
254 additional MAP testing. While the sensitivity, specificity, accuracy and positive predictive
255 value of raised SCC for predicting MAP seroconversion is probably insufficient diagnostically,
256 it may aid in the surveillance in some herds. A prospective cohort study with more frequent
257 (weekly) SCC and Johne's milk ELISA testing will allow us to evaluate these dynamics better.
258 In such proposed work, it would be advisory to evaluate the Th1/Th2 preference of the

peripheral blood monocytes to evaluate whether the two local immune responses in the intestine and the udder are linked centrally, or not.

To conclude, this study found cows infected with MAP have increased SCC close to the time that they first became seropositive. This is identified for the periods before and at the point seroconversion was first diagnosed, suggesting that an increased SCC might be predisposing cows towards this progression of MAP. Due to the nature of the paratuberculosis and the study design used, a possible causative pathway cannot be supported; a temporal relationship, however, can be suggested.

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412 **Table 1: Average somatic cell count (x10^{3}cells/ml) in case and control cows by antibody**

413 **response group, number of cows presented in brackets. Time0: recording when the first**

414 **positive antibody result occurred. *: different case vs. control p<0.05. #: different SCC**

415 **level over the reported time period p<0.05.**

Average somatic cell count (x10^{3}cells/ml)	Antibody Response Group					
	High (44)		Progressive (15)		Transient (53)	
	Control	Case	Control	Case	Control#	Case
Previous recording	54	105*	146	142	75	110*
Time0	57	127*	93	126	112	159
Subsequent recording	64	102	77	100	98	86

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418 **Table 2: High somatic cell count prevalence in case and control cows by antibody response**
419 **group, number of cows presented in brackets. Time0: recording when the first positive**
420 **antibody result occurred. *: different case vs. control p<0.05. #: different number of high**
421 **SCC events over the reported time period p<0.05.**

Percentage of high SCC ($>200x10^3$cells/ml)	Antibody Response Group					
	High (44)		Progressive (15)		Transient (53)	
	Control	Case	Control	Case	Control#	Case#
Previous recording	11.4%	36.4%*	46.7%	33.3%	20.8%	37.7%
Time0	6.8%	36.4%*	26.7%	33.3%	39.6%	47.2%
Subsequent recording	13.6%	31.8%*	33.3%	33.3%	20.8%	26.4%

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