Research paper

Effect of days in milk and milk yield on testing positive in milk antibody ELISA to Mycobacterium avium subsp. paratuberculosis in dairy cattle

Søren Saxmose Nielsen*, Nils Toft

Department of Large Animal Sciences, University of Copenhagen, Grønnegårdsvej 8, DK-1870 Frederiksberg C, Denmark

A R T I C L E   I N F O

Article history:
Received 13 March 2012
Received in revised form 8 May 2012
Accepted 10 May 2012

Keywords:
Days in milk
ELISA
False-positive
Paratuberculosis

A B S T R A C T

Milk samples are becoming more used as a diagnostic specimen for assessment of occurrence of antibodies to Mycobacterium avium subsp. paratuberculosis (MAP). This study assessed the effect of days in milk (DIM) and milk yield on testing positive in a commercial MAP specific milk antibody ELISA among 222,774 Danish Holstein cows. Results showed that odds of testing positive on 1–2 DIM were 9–27 times higher than the rest of lactation, where the chance of testing positive varied less. The reason is most likely a high concentration of non-specific antibodies in colostrum. Consequently, samples from the first couple of DIM should be excluded from MAP testing until further information on their significance is established. Milk yield also had a significant effect on odds of testing positive due to its diluting effect. Inclusion of milk yield in the interpretation of test results could improve the diagnostic value, resulting in more predictable patterns corresponding to progression of infection.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) causes significant losses to the dairy cattle industry (Ott et al., 1999). Consequently, several dairy producing countries have implemented programs to reduce the effects caused by MAP infections (Kennedy and Nielsen, 2007; Nielsen, 2009). Use of diagnostics in these programs can include agent based tests, such as bacteriological culture or PCR on fecal samples, or detection of MAP specific antibodies using ELISA on serum or milk samples (Nielsen and Toft, 2008). Because MAP infections are chronic, sensitivity and specificity of MAP diagnostics are a function of infection stage. Choice of diagnostic method therefore depends on the purpose of testing (OIE, 2011). Once a purpose has been defined, a test which can detect a specific target condition related to this purpose should be selected to optimize utility of the test results. However, costs and logistics related to testing also play a major role. Use of milk samples for detection of antibodies has the advantage that sampling can be automated via milk recording schemes, where samples are already shipped to a laboratory.

Milk and serum IgG levels are affected by stage of lactation, with early and late stage lactation samples resulting in higher odds of testing positive compared to mid-stage lactation in milk, whereas late–stage lactation yield highest odds in serum (Nielsen et al., 2002; Lombard et al., 2006). It has been speculated that these effects are primarily due to: (a) high IgG1 and IgG2 in colostrum samples, i.e. the first few days of lactation; (b) dilution of milk samples because cows in different stages of lactation produce different levels of milk, and (c) progression of MAP infection. However, sample sizes have so far been insufficient to describe day-by-day variation in test-responses.

The primary objective of this study was to estimate the effect of day of lactation on the chance of testing positive in
a commercial ELISA for detection of MAP specific antibodies in milk from dairy cows, while correcting for progression of infection (modeled using parity as a proxy). A secondary objective was to determine if this effect was affected by milk yield as a dilution factor.

2. Materials and methods

2.1. Herds and animals

All herds enrolled in the Danish control program on paratuberculosis (Nielsen, 2007) in the period 15 October 2008–19 May 2011 were initially included in the study. “Enrolment” implied that four times per year all milking cows in a herd were tested. A total of 1364 of the approximately 4500 dairy herds in Denmark contributed data for such whole-herd screenings, although not all herds contributed samples for the entire study period. Within these herds, 318,227 cows provided 1,226,761 milk samples, which were tested for antibodies to MAP (see below).

Data on milk antibody result, test-date, calving date and milk yield on the test-date were obtained from the Danish Cattle Database (Knowledge Centre for Agriculture, Aarhus, Denmark). The within-herd test-prevalences in these herds ranged from 0 to 33%, with a lower quartile of 2%, a median of 3.7% and an upper quartile of 6%.

2.2. Detection of antibodies

Milk samples were collected via the Danish milk recording scheme, and through this scheme herds enrolled in the Danish MAP control program are automatically identified, when a herd is due for testing of antibodies to MAP. Milk samples were analyzed using the commercial test ID Screen® Paratuberculosis Indirect ELISA kit according to instructions of the manufacturer (ID Vet, Montpellier, France) at Eurofins Stein’s Laboratory (Holstebro, Denmark). This kit detects MAP specific IgG1 and IgG2. A sample was considered positive at a sample-to-positive ratio of 0.20 as recommended by the manufacturer.

2.3. Statistical analyses

To reduce the complexity of the statistical analyses, only Holstein cows (222,774 cows (70%)) were included and only one sample from each cow was selected at random and used in the study. Random selection was done using a random number generator in SAS version 9.2 (SAS Institute, Cary, NC, USA). The selected population was then characterized by the distributions of parity, DIM and milk yield (kg). Samples from cows with more than 500 DIM were excluded, because these were rare in most herds.

A generalized linear mixed model was used to model the probability of testing positive in the milk ELISA as a function of the covariates DIM, parity and milk yield, including herd as a random effect to account for within-herd clustering. The following strategy was used to model the covariates:

(a) DIM was the primary covariate of interest and consequently the others were included to control for their potential effect. First, DIM was grouped into the following intervals: 1, 2, 3, 4, ..., 30, 31–40, 41–50, ..., 491–500. This grouping was chosen because previous results have suggested that the first weeks of lactation strongly influence the results of the milk ELISA, and consequently these need closer scrutiny (Nielsen et al., 2002). The results of the effect of these DIM groups were inspected for linearity in the regression analysis. This inspection (see later) suggested that there was an approximate linear effect of DIM from day 6 to day 350 and from day 350 to day 500. Therefore, the DIM groups 1–6 were retained, but DIM > 6 were included as piece-wise linear effects in each of the two intervals.

(b) Parity was grouped into parity 1, parity 2 and parity >2, because previous results suggested that the effect of parity wanes around parities 2 and 3. This covariate was included to account for progression of infection along with DIM. The model also included an interaction between DIM/DIM group and parity group to allow for different patterns within each parity group (Nielsen et al., 2002).

(c) Milk yield was included as a continuous variable centered around the grand mean (29.0 kg milk) and initially included as 1st, 2nd, 3rd, 4th and 5th order polynomials with subsequent reduction of non-significant terms. Furthermore, the effect of milk yield was modeled as a categorical variable, where the cows were grouped into 10 equally sized groups based on the 10 percentiles of milk yield.

(d) The effect of inclusion of milk yield was then illustrated comparing results from a model including and one excluding milk yield. For this analysis, only data from 7 to 500 DIM were used, because recordings from DIM 1 to 6 may obscure the results if included.

(e) The intra class coefficient (ICC) was estimated for the final model based on the herd variance ($\sigma^2_N$) using the formula: $\sigma^2_N = \sigma^2 + (\pi^2/3)$.

The results of the model were presented as population averaged odds ratios (Dohoo et al., 2009). The analyses were carried out using the Glimmix procedure in SAS version 9.2 (SAS Institute, Cary, NC, USA). ‘Statistical significance’ was deemed at P-values of <0.05 based on the likelihood ratio test. Model fit was assessed by (a) model convergence, i.e. obtaining a solution from the Glimmix procedure; (b) the Pearson $\chi^2$ statistic, as a means of judging unexplained extra Binomial variation, and (c) preference of a parsimonious model (when conclusions were unchanged) constructed using the above-mentioned strategy.

3. Results

The population used had the following characteristics: the parity distribution was: parity 1: 120,266 (54%); 2: 53,307 (24%); >2: 49,201 (22%). The milk yield followed an approximate normal distribution with mean 29.0 kg and a standard deviation of 8.4 kg. The DIM distribution is shown in Fig. 1. There were 152 observations on DIM 1 and 294
on DIM 2, with the remaining days including between 300 and 722 samples per day.

Milk yield, parity group and DIM all had a significant effect on the odds of testing positive in the milk ELISA. The initial analyses suggested that DIM was linear from approximately 6 to 350 DIM and again from 350 to 500 DIM, and further modeling therefore included linear effects of DIM in these two intervals. Milk yield included as a categorical variable gave a better model fit than when included as polynomials. There was significant interaction between parity group and the two linear effects of DIM, but not between DIM-groups 1–6 and parity group. The final model thus included the effect of milk yield as a categorical dilution factor, DIM-groups 1–6 as categorical factors, and DIM as piecewise linear effect within each parity group, allowing the linear effect to change at 350 DIM (Fig. 2). The intra class coefficient for herd was estimated to 0.11 based on a herd-level variance of 0.38.

The odds of testing positive on DIM = 1 was 27 times higher than the odds for cows in DIM 7–500. From 6 DIM, the odds were lower than 2 for the rest of the lactation, and consequently exerted less variation than in the initial 1–5 DIM. The effect of DIM increased throughout parity 1 and continued into parity 2, where the odds leveled out and seemed stable after 350 DIM in parity 2 and onwards (Fig. 2). However, this was only the case if the odds were corrected for milk yield (Fig. 3). Exclusion of milk yield affected the chance of testing positive by up to 100% and resulted in more variation across lactation.

4. Discussion

Stage of lactation had a major effect on the odds of testing positive in milk antibody ELISA, particularly during the first days of lactation, where the odds were 3–27 times higher than mid-lactation. This is not surprising
considering that colostrum generally has a high overall (non-pathogen specific) content of both IgG1 and IgG2 (Butler, 1983; Baumrucker et al., 2010). Concentrations of IgG1 and IgG2 in colostrum whey have been estimated to 46 and 2.9 mg/ml, respectively, whereas concentrations in milk sampled after more than 14 DIM were only 0.58 and 0.055 mg/ml, respectively (Butler, 1983). The concentration of IgG2 has also been estimated to be at the same level at 0 DIM as the concentration of IgG1 at 180 DIM (Guidry et al., 1980). Therefore, it is likely that MAP specific IgG2 reaches detectable levels in colostrum, whereas the concentrations are too low in milk obtained later in lactation.

MAP specific IgG2 is primarily associated with early stage MAP infections with pre-dominant cell-mediated immune-responses. In contrast, MAP specific IgG1 is generally considered to be associated with a shift from cell-mediated immunity toward humoral immunity and MAP shedding (Koets et al., 2001). Consequently, detection of MAP specific IgG1 antibodies would suggest that such a shift has occurred, and detection of this shift would indicate that the cow is becoming a MAP shedder. However, the increased odds of testing positive may not be related to detection of MAP specific IgG1, but rather that IgG2 suddenly reached detectable levels in colostrum. Occurrence of IgG2 may suggest that the test-positive animal has been exposed to MAP and that the animal is potentially infected, but not that the unfortunate shift toward humoral immunity and MAP shedding has occurred (Koets et al., 2001). However, if the increased odds of testing positive are caused by IgG2 indicating early-stage infection, and the purpose of testing is to detect late-stage infection (MAP shedders), then a positive result would be a false-positive. On the other hand, if IgG2 was detected as an indicator of MAP infection more generally, then colostrum would be an ideal diagnostic specimen.

Although the above-mentioned explanation might seem appealing from a diagnostic perspective, it is more likely that the positive test results in early lactation are caused by binding of non-specific protein to the ELISA wells. This may occur when concentrations of protein are high, and has also been described for rotavirus neutralizing antibodies in colostrum and milk (Archambault et al., 1988). In our study, the majority of the animals testing positive the first days in lactation were test-negative on the follow-up test (data not shown), but no specific patterns could be identified and life-time data for these cows would be required to determine the significance of this early-stage lactation test-positivity. Such data are currently not available. An alternative could be to use the within-herd prevalences, if they were sufficiently different. It would then be expected that the proportion of test-positive in early lactation would be similar in low and high prevalence populations if the reactions were non-pathogen specific. However, the within-herd prevalence distribution was relatively narrow with 75% of herds having <6% test-positive and consequently we did not pursue this approach.

Throughout the remaining part of lactation, variation was much less pronounced than in the first 1–5 DIM, but still quite marked, e.g. with odds of testing positive 4 times higher in the end of parity 1 compared to the early lactation (Table 1). The primary reason for this increase is progression of infection, i.e. a pathogenetical phenomenon. It would be ideal if this effect could be separated from technical characteristics such as the effect of dilution. Fig. 3 illustrates nicely that by correcting for milk yield, the effect of DIM within each parity becomes almost linear throughout the entire parity. Furthermore, the odds at the end of parity 1 is more or less similar to the odds at the beginning of parity 2, with similar features for parity 2 and parity >2. Therefore, correcting for milk yield would ease interpretation
of milk antibody ELISA results, separating technical and pathogenetical aspects. A downside to the interpretation of the present data was lack of a true infection and antibody status of the animals tested. Such information is rarely available for MAP infections and never in large datasets.

In conclusion, the present data suggest that particularly DIM, and partly milk yield cannot be ignored when interpreting ELISA results. Under practical circumstances such as in control schemes, samples from DIM 1 to 5 should be excluded until further knowledge of their significance is established, while inclusion of milk yield to account for effect of dilution could result in a smoother prediction of test-results, which would correspond to progression of infection.

References


