Bulk tank milk ELISA for detection of antibodies to *Mycobacterium avium* subsp. *paratuberculosis*: Correlation between repeated tests and within-herd antibody-prevalence

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**ABSTRACT**

Detection of bulk tank milk (BTM) antibodies using ELISA (BTM-ELISA) may constitute an inexpensive test for surveillance of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in dairy cattle herds provided that the test is accurate and consistent. The objectives of this study were to determine: (a) the correlation between repeated BTM reactions; and (b) the association between the BTM antibody ELISA-level and the within-herd prevalence of antibody-positive cows.

Eight BTM samples per herd and approximately four milk samples per lactating cow per herd were collected from each of 108 Danish Holstein herds over a period of one year. All samples were tested using a commercial indirect ELISA for detection of MAP specific antibodies. The individual cow’s results were dichotomised and used to estimate the within-herd antibody prevalence at each test-date. These prevalences were then combined with the ELISA reading on the BTM test-date closest to the cow-level test-date.

A mixed-effect analysis of covariance with autoregressive type 1 correlation structure was carried out using the log-transformed BTM-ELISA results as outcome. This model was used to assess the correlation between repeated tests with and without correction for within-herd antibody prevalence.

The repeated BTM-recordings were highly correlated with a correlation of 0.80 between samples collected 1.5 months apart. The within-herd antibody prevalence significantly influenced this estimate ($p < 0.0001$), which dropped to 0.60 when corrected for the within-herd antibody prevalence. Although the test-results were relatively consistent and correlated with the within-herd prevalence, the magnitude of the test-values makes it difficult to use the BTM-ELISA for surveillance of MAP infections in practice.

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1. **Introduction**

Bulk tank milk (BTM) testing is a frequently used tool for surveillance, monitoring or control of infectious diseases in dairy cattle, e.g. bovine herpes virus-1 (BHV-1, Nylin et al., 1999; Ståhl et al., 2002), enzootic bovine leukosis (EBL, Reber et al., 2012), bovine virus diarrhea (BVD, Bitsch and Rønsholt, 1995; Valle et al., 2001) and *Salmonella* Dublin (Warnick et al., 2006). These infections have characteristics rendering them ideal for surveillance using BTM in populations, where vaccination has not been carried out. This includes a relatively fast sero-conversion of infected animals or relatively high within-herd prevalences. BTM tests for BVD, BHV-1 and S. Dublin have relatively high sensitivities and have been used for surveillance (Beaudeau et al., 2001a; Nylin et al.,
1999; Warnick et al., 2006), and an association between the within-herd prevalence of antibody-positive cows and the BTM-antibody level has also been established for BVD (Niskanen, 1993; Beaudou et al., 2001b) and S. Dublin (Nielsen and Ersbøll, 2005). Acute viral infections such as BVD virus and BHV-1 generally result in a relatively fast sero-conversion, frequently with a large proportion of the herd sero-converting leading to high concentrations of antibodies in the bulk tank milk. The BTM-antibodies then gradually disappear with the removal of infected animals (Houe, 1999).

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections are subject to control or monitoring in several major dairy producing countries (Kennedy and Nielsen, 2007), because of a major impact of MAP infections on farming economy (Ott et al., 1999). BTM-ELISAs for MAP infections appear to be relatively sensitive and specific for detection of herds with high within-herd prevalences (Nielsen et al., 2000; van Weerden et al., 2007). However, the BTM ELISA tests seem to be quite sensitive to selection of a cut-off value used to deem a herd infected, and low-prevalence herds may go undetected. The within-herd prevalence in MAP infected herds is often low (Nielsen and Toft, 2009), many infected cattle do not have antibodies (Collins et al., 2005), and the antibody levels may vary significantly within infected cows (Nielsen et al., 2002). These are some of the challenges faced if BTM testing should be used. However, the correlation between the BTM response and the within-herd prevalence has not previously been determined for MAP infections and it is not known how the test-responses vary over time.

The objectives of this study were therefore to determine: (a) the correlation between repeated bulk tank milk reactions; and (b) the association between the bulk tank milk antibody ELISA-level and the within-herd prevalence of antibody-positive cows.

2. Materials and methods

2.1. Herds and animals

A total of 110 herds were selected randomly among herds fulfilling the following criteria in November 2011: (a) be Danish dairy herds, i.e. delivering milk to a dairy processing plant in Denmark (n = 3920); (b) be enrolled in the Danish control programme on paratuberculosis (n = 1139) (Nielsen, 2009a); (c) perform four annual whole-herd screenings using milk antibody ELISA (as compared to four annual partial screenings) (n = 523); (d) have Danish Holstein cattle as the main breed; and (e) no MAP vaccinated cattle should be present in the herd.

2.2. BTM sampling

BTM samples were collected via the Danish milk quality system, where the specific sampling date is not known to the dairy farmer. Bulk tank milk samples were collected on eight predefined sampling dates approximately 1.5 months apart, covering the period March 2012 to February 2013. The samples were all sent to Eurofins Steins Laboratory (Holstebro, Denmark) and tested for MAP antibodies (see below).

2.3. Cow-level sampling

Data from the Danish control programme on paratuberculosis were used for estimation of the within-herd antibody prevalences of paratuberculosis. In this programme, milk samples are collected at the routine milk recordings also assessing parameters such as milk yield, fat, protein and somatic cell counts. Therefore, the cow-level sampling dates were predetermined by the date of this recording, without the possibility of sampling the same date as the BTM milk test-date. Two of the 110 herds ceased participation in the control programme prior to the start of sampling and consequently no cow-level data were available from these herds and they were excluded from the study. All lactating animals were tested at the herd-screenings occurring in each herd. Among the remaining 108 herds, sampling was conducted 5 times in 78 herds, 4 times in 29 herds and 3 times in 1 herd. The cow-level sampling date closest to a specific BTM date was selected for further comparisons.

2.4. Antibody ELISA

All milk samples (bulk tank and individual cow’s milk) were tested using the ID Screen® Paratuberculosis Indirect Screening test according to the description of the manufacturer (ID-Vet, Grabels, France). This test is a Mycobacterium *phlei* absorbed ELISA for detection of MAP specific IgG. The resulting optical density (OD) values were transformed to sample-to-positive (S/P) ratios to take into account a set of positive and negative controls using the formula:

\[
\text{S/P\%} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}}}{\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}} \times 100\%
\]

Cow-level samples were considered positive at S/P% > 15 as recommended by the manufacturer, and the within-herd antibody prevalence was estimated as the proportion of test-positive among all animals tested on a specific test-date. Bulk tank milk results were kept on a continuous scale, but the cut-off recommended by the manufacturer for BTM is also 15 S/P%.

2.5. Statistical analyses

Descriptive statistics were done by plotting the distribution of BTM S/P% overall, and depicting the S/P% for each herd over time. The latter was done stratified in four groups based on the median S/P-value of the herd to better visualise the stability of the S/P-values within a herd. Furthermore, Spearman’s rank correlation coefficients were calculated to determine the crude correlation between the repeated observations. Finally, the raw S/P% values were plotted as a function of the within-herd antibody prevalences.

For the analytical statistics, the following model was used:

\[
\log(Y_{ij} + 0.5) = \mu + \beta X_i + \epsilon_{ij} \text{ with } \text{cov}(Y_{ij}, Y_{ik}) = \sigma^2 \rho^{j-k}
\]
where \( Y_{ij} \) is the value of BTM S/P for the \( i \)th herd at the \( j \)th sampling, with 0.5 added to avoid taking the log of 0; \( \mu \) was the intercept, \( \chi_i \) the within-herd antibody prevalence of ELISA-positive animals in the \( i \)th herd; \( \varepsilon_{ij} \) were normally distributed residuals \( N(0, \sigma^2) \) and the covariance between samples dates within the herd followed a first order autoregressive model AR(1) with parameter \( \rho \). The following covariance structures were also assessed but were deemed with lower fit based on Akaike’s Information Criterion: compound symmetry, Toeplitz, spatial power, first-order moving average and first-order antedependence. The natural logarithm to the S/P-values was used to achieve a normal distribution of the residuals. First, the between-test date correlation was estimated using a model without the fixed effect. Then the within-herd prevalence, \( \chi_i \), was included to determine the association to the log-transformed S/P values and to determine the autocorrelation in the presence of this parameter. All analyses were performed using the Mixed procedure in SAS v. 9.3 (SAS Institute, Cary, NC, USA).

3. Results

The herd size distribution (on the bulk tank milk sampling days) was as follows: minimum: 11 cows, 1st quartile: 124 cows, median: 158 cows, 3rd quartile: 229 cows, and maximum: 616 cows. The distribution in the length of the period between cow and bulk milk sampling was: minimum: 0 days, 1st quartile: 12 days, median: 23 days, 3rd quartile: 35 days and maximum: 104 days. The distribution of ELISA-values had a minimum of 0 S/P%, 1st quartile of 3 S/P%, median of 6 S/P%, 3rd quartile of 12 S/P%, and a maximum of 111 S/P% (Fig. 1). The herd-profiles constructed of repeated S/P-values within a herd, and stratified based on the quartiles of the within-herd-median S/P, are shown in Fig. 2a–d, with the resulting Spearman’s rank correlation coefficients shown in Table 1. The number (percentage) of herds with 1, 2, 3, . . . , 8 positive samples were: 1 positive: 43 (40%), >1 positives: 34 (31%), >2 positives: 25 (23%), >3 positives: 15 (14%), >4 positives: 10 (9%), >5 positives: 8 (7%), >6 positives: 6 (6%), and 8 positives: 3 (3%).

The distribution of the within-herd apparent prevalences was: minimum: 0%, 1st quartile: 2.0%, median: 4.3%, 3rd quartile: 6.9% and maximum: 31%. The BTM S/P% plotted as a function of within-herd prevalences are shown in Fig. 3. The BTM S/P% was significantly associated with the within-herd prevalences (\( p < 0.0001 \)) with a regression coefficient \( \beta \) of 12.2, as shown in Fig. 3. The between-test date autocorrelation was 0.80 (95% confidence interval: 0.76–0.83) when the within-herd test prevalence was not taken into account by the model used, while it was 0.60 (95% confidence interval: 0.53–0.67) when the within-herd prevalence was included in the model. The residuals of both models were deemed to be independent and identically distributed Normal (0, \( \sigma^2 \)).

4. Discussion

The present study demonstrated that the BTM antibody level was significantly associated with the within-herd prevalence of antibody positive animals and repeated measurements of BTM antibody level were relatively highly correlated. Herds with a high median S/P value were also those with the most pronounced fluctuation in the S/P-values (Fig. 2). At first sight, this might be promising for use of the BTM test for surveillance or control of MAP infections. Although it appears to be promising, it really is not, because the BTM test does not really detect the infected herds, as will be explained in detail in the following paragraphs.

A useful surveillance test should be able separate infected from non-infected herds and the test results should be consistent positive as long as the infection is present (Christensen and Gardner, 2000; Greiner and Gardner, 2000). For a semi-quantitative test such as the indirect ELISA, this could be reflected in a bimodal distribution of the BTM ELISA values. Such are BVD BTM-ELISAs, which also use the entire range of the ELISA scale, and exhibit a nice, although not perfect, separation into herds with and without BVD virus infected animals (Beaudeau et al., 2001a,b; Bitsch and Rønsholt, 1995; Houe, 1994). The present MAP BTM test essentially appears unimodal (Fig. 1). The maximum for the BTM ELISA was 111 S/P% with the 3rd quartile being 12 S/P% (Fig. 1), while the maximum for the individual samples was 370 S/P% (data not shown). Consequently, the possible range of values for the BTM ELISA was not really used, despite an expected high prevalence (see below). Few observations (2%) were above 40 S/P% and not consistently so (Fig. 2d). This is problematic, because the practical utility is reduced if the surveillance results are in a relatively narrow range with an apparent unimodal distribution. The manufacturer may recommend a cut-off of 15 S/P% for deeming a herd positive, but the basis for this dichotomisation is more or less non-existent.

If separation into a likely infected and a potentially non-infected population is possible, estimates of the within-herd prevalence could help in selecting an appropriate control scheme for a specific herd. The results
Fig. 2. *Mycobacterium avium* subsp. *paratuberculosis* specific bulk tank milk antibody ELISA profile for 108 Danish dairy herds tested up to 8 times over 1 year, stratified by in quartiles of the median ELISA S/P-ratio within each herd. Herds with median in (a) lower quartile, (b) 2nd quartile, (c) 3rd quartile, and (d) upper quartile. The dotted line indicates the recommended cut-off from the manufacturer.

presented here suggest that increasing within-herd antibody prevalence will result in an exponentially increasing BTM S/P-value. However, the predictive capability of the BTM test on the within-herd prevalence was not established. Antibodies on cow-level accumulate to herd-level, hence herd-level antibodies are the result of cow-level antibodies. A model suggesting the reverse relationship, i.e. herd-level predicting cow-level, would be biologically meaningless. To establish the within-herd prevalence, cow-level screening would still be required.

Besides being able to discriminate between infected and non-infected herds, the test should be consistently positive or negative if the herd is tested repeatedly and if the herd-status is the same. MAP infections are chronic and eradication of MAP from infected herds does not usually occur in the time span of one year as the study period was here. Therefore, the test-responses should also be consistent in most herds. This consistency was assessed based on between-sampling correlations, as well as the proportion of samples above/below the recommended BTM S/P cut-off over time. The between-sampling correlation depends on the between-herd prevalence, because non-infected herds would not be expected to have MAP specific antibodies. In these non-infected herds, the variation in ELISA responses

<table>
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<tr>
<th>Parameters</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
<th>Sample 7</th>
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<td>0.72</td>
<td>0.71</td>
<td>0.65</td>
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would be of a different nature than in the infected herds, e.g. laboratory associated (Nielsen, 2002). In both infected and non-infected herds, non-MAP specific antibodies may occur due exposure to environmental mycobacteria and thereby contribute to the ELISA response (Osterstock et al., 2007). It could be argued that a good test does not exhibit variation beyond that associated to antibodies, but that is difficult to achieve for MAP specific ELISAs, which are often pushed to the edge of their performance, so they are able to detect low antibody concentrations. However, the primary variation of indirect ELISA responses should be caused by antibodies, not other factors. The between-herd prevalence is notoriously difficult to estimate (Nielsen and Toft, 2009), but the between-herd prevalence in Denmark is very likely above 50% and probably around 85% (Nielsen, 2009b) and 75% of herds had more than 2% test-positive cows in the present data. A useful screening test would therefore be expected to detect more than 50% or perhaps even more than 75% of the herds, especially if low-prevalence herds should also be detected. Only 34% of the herds had multiple test-values above the manufacturer’s recommended cut-off (Fig. 2), and very few (3%) were persistently above the cut-off. Therefore, the sensitivity was deemed to be low and the persistency or consistency of the responses equally so. This does not correspond to a correlation of 0.80 being interpreted as “relatively high”. The uncorrected correlation of 0.80 between the recordings was at the same level as has been demonstrated for Coxiella burnetii antibody detection (Nielsen et al., 2011), where correlations of 0.85–0.87 were recorded for BTM samples collected approximately 1 month apart. However, the interpretation of this correlation is tricky, because factors unrelated to pathogen specific antibodies may result in a high correlation in case of a low prevalence of antibodies. Therefore, the difference in the prevalence-corrected and un-corrected correlation is actually the interesting part. The prevalence-corrected correlation was still relatively high (0.60) suggesting that a lot of the variation was due to other factors than MAP specific antibodies. There are basically two sources of variation resulting in “false-positive” reactions: laboratory factors and herd/animal-related factors. Laboratory factors are generally poorly characterised, but known to be able to account for significant parts of ELISA variation (Nielsen, 2002). Animal-related factors can be due different binding capacities of the lacteal secretions, e.g. colostrum has a different affinity to ELISA-plates than milk (Zervens et al., 2013) or exposure to mycobacteria other than MAP may in some herds result in production of cross-reacting antibodies (Osterstock et al., 2007). Both would essentially be likely to contribute to both the within-herd antibody prevalence as well as the BTM test reaction, while the animal and BTM tests are the same. The factors causing false-positive reactions are poorly characterised, and will therefore in the following be referred to jointly as “FP-factors”.

The level of false-positives is usually described by the specificity, which for the present ELISA used for cow level samples has been estimated to 0.987 (Nielsen et al., 2013). This means that in a herd with the median within-herd prevalence of 4.3%, 1.3% of the test-positive samples could be false-positives, i.e. that median within-herd prevalence of cows infected with MAP and with MAP specific antibodies might only be 3%, not 4.3%. However, we do not know if FP-factors affect both the BTM S/P value and the cow-level S/P-value, or just one of them. FP-factors are likely affected differently when a cow-level milk sample is diluted in the BTM, e.g. high concentrations of cross-reacting antibodies in a single cow-sample may still be detected in the BTM ELISA, but the effect of
a colostrum diluted sample may be gone, if this effect was not due to MAP specific antibodies. Furthermore, cows with high concentrations of *Salmonella* Dublin antibodies has been shown to contribute more to BTM S/P values than cows with low concentrations of antibodies (Nielsen and Erbsbøll, 2005), but this feature is not really captured in the dichotomisation process when we establish the within-herd prevalence. However, changing the cow-level cut-off for prevalence estimation from 15 S/P% to 150 S/P%, which would effectively increase the specificity and take the concentration issue into account, did not really affect the correlation between BTM S/P values and the within-herd prevalences (results not shown). Therefore, even with this ‘removal’ of most non-specific values on cow-level, the correlation remained constant. Correction for FP-factors does not seem to be possible, and separation between FP factors and dilution effects are essentially impossible. The sensitivity of the antibody ELISA is also low, but this should not affect the relation between BTM and cow-level reactions, because lack of antibodies would affect both the BTM and the individual cows’ sample. The apparent prevalences were used because correction for the test sensitivity would only make a relative correction of all parameter estimates.

Despite the high statistically significant correlation between the within-herd antibody prevalence and the BTM S/P-values, the uncertainty was huge (as indicated by the wide 95% confidence band in Fig. 3), and the practical value of BTM antibody recordings is therefore limited. A problem may be the lack of reaction of the majority of samples, despite the association between the within-herd prevalence and the BTM S/P-values. A major part of the correlation between samples seems to be due to other factors than MAP specific antibodies. Therefore, one way of increasing the sensitivity is to increase the frequency of sampling, but still less than 25% of herds had more than 2 of the 8 samples positive, while the within-herd prevalence was expected to be above 50%. A sampling scheme with 8 annual samples may therefore miss a major proportion, assuming that the prevalence estimates are correct. A high but not perfect correlation between repeated samples increases the sensitivity, but in the present case the practical gain in sensitivity is limited. If the BTM should be used in practice, communication of fluctuating results and decision making resulting from the testing would pose a challenge to the decision makers.

5. Conclusion

To conclude, this study demonstrated that the ELISA results of repeated BTM samples collected 1.5 months apart were highly correlated and the BTM test response was significantly associated with the within-herd prevalence of antibody positive cattle. However, despite the clear statistical associations, the practical utility of BTM testing for MAP seems limited, because the BTM test-responses are in a relatively narrow range of the test, and uncertainty associated with the test-responses will likely make communication to users a challenge unless carefully used in a diagnostic test strategy.

Acknowledgement

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References


