

Analysis of Antibody in Individual and Bulk Milk Samples is a Useful Tool for Investigation of Johne's Disease

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Abstract

There is a need for improved cost-effectiveness when assessing a herd for the presence of Johne's disease. The aim of this study was to determine whether this could be addressed by testing individual milk rather than serum samples, and screening targeted animals and bulk milk instead of an entire herd. The performance of an ELISA kit routinely used in our laboratory for quantifying serum levels of antibodies to *Mycobacterium avium paratuberculosis* (*Map*) was assessed for the analysis of milk samples. To this end, *Map* antibody titres were quantified in matched serum and milk samples taken from the same animal. Thereafter, bulk milk titres from herds infected with *Map* and from herds judged to be free of infection by sampling target animals within the herd were compared. There was 95% agreement between serum and milk ELISA results ($r^2=0.84$). Targeted sampling results agreed with the clinical history in all except one farm. There was a significant difference between the bulk milk titres from known infected herds and assumed negative herds ($p=0.004$). Analysis of serial dilutions of antibody positive milk samples in negative milk indicated that bulk milk sampling could be used to detect herds with a prevalence of antibody positive animals as low as 3% using the individual milk cut-off level. All bulk milk samples from negative herds had a percentage positive result of ≤ 10 , suggesting that a lower positive cut-off level can be implemented for interpretation of bulk milk samples than for individual milk samples. Together, the results indicate that milk is a suitable medium for the quantification of *Map* antibodies by ELISA, targeted sampling has potential as a herd screening test and bulk milk sampling may be useful in monitoring herd infection level. Both of these latter techniques carry the limitation that they may be insufficiently sensitive to detect herds with a low prevalence of disease.

Keywords

Map, Johne's, paratuberculosis, targeted sampling, milk, bulk milk, ELISA, *Mycobacterium paratuberculosis*

Introduction

Johne's disease, as a result of infection with *Map*, presents challenges for the vet and the farmer alike, both in its diagnosis and its control. Commercially available ELISAs designed to quantify antibodies to *Map* are known to have a low sensitivity (Collins 2005). Despite this, the test is cheap, readily performed and gives a rapid result so that it remains a useful tool in the surveillance for and control of Johne's disease (Klausen 2003, Caldow 2004). Although the sensitivity is low, it increases as disease progresses (Collins 2002, Sweeney 1995) thus the ELISA can be of particular value in detecting animals that are likely to be bacterial shedders and constitute a risk for the remainder of the herd.

Milk samples are routinely used for quantifying antibody titres to other bovine diseases but the assessment of *Map* antibodies in milk is less well established. In addition, the

ELISA is not generally considered sensitive enough to identify infected herds using bulk milk testing unless the herd has a high prevalence of disease (Institute Pourquier Montpellier, France), although one commercially available ELISA kit claims bulk milk analysis can be used to classify herds into “unsuspected” or “low prevalence” or “high prevalence” categories (Svanova, Uppsala, Sweden).

A number of cattle health schemes are available which provide ‘accredited free’ and ‘screening and eradication’ programmes for Johne’s disease under the auspices of CHeCS. These programmes are expensive for the farmer to implement as they involve annual whole herd testing and may not be worthwhile for dairy farmers in the current financial climate (Stott 2005). There is, however, a need for replacement stock that is likely to be free of the disease as these are the major source for introduction of infection into a herd. Targeted sampling and bulk milk screening may be a cheaper yet sufficiently effective way of addressing this demand. Although not as secure as buying from an ‘accredited free’ herd, farms with repeatedly negative bulk milk samples and negative screening of cows that are sick or have reduced productivity may have a lower prevalence of disease and are thus a more secure source than herds with a clinical Johne’s problem.

The aims of this study were to determine whether the ELISA used in our laboratory to quantify antibodies to *Map* gave consistent results between serum and milk samples and if targeted cow screening and bulk milk sampling could be used to identify farms with Johne’s disease.

Materials and Methods

The ELISA used for all tests was a commercially available kit (Institute Pourquier, Montpellier, France). Following the instructions of the kit, the results of the *Map* ELISA are reported as percentage positive relative to the positive control, designated S/P. The cut-off values recommended by the kit were used for the interpretation of serum and individual milk samples. For serum these were: negative – $S/P \leq 60$; doubtful – S/P is between 60 and 70; positive – $S/P \geq 70$, and for individual milk these were negative – $S/P \leq 30$; doubtful – S/P is between 30 and 40; positive – $S/P \geq 40$.

To study the correlation between milk and serum *Map* antibody titres by ELISA, serum and matched milk samples were obtained from 155 dairy cows from a number of farms. Bulk milk samples were obtained from 26 dairy herds. Eight herds were known to be infected with Johne’s disease prior to the study, and 1 was known to be uninfected (closed herd, monitored free since 1997). Seventeen herds had an unknown Johne’s status, although 3 of these had experienced a single case in bought-in animals; none of the others had a history of the disease. To allow comparison of bulk milk results in positive and negative herds, Johne’s status was defined by analysis of ‘targeted’ individual milk and matched serum samples. The aim was to identify 6 animals in each herd with a history suggestive of Johne’s disease such as weight loss or reduced productivity for targeted sampling, although in some herds different numbers of animals were sampled. If no positive results were obtained in the targeted samples, the herd was designated ‘negative’ for Johne’s.

The usefulness of bulk milk tank sampling in detecting infected herds was further assessed by making serial dilutions of 6 individual milk samples with positive *Map* antibody titres (S/P range from 47 to 266) in milk that was negative for Johne’s antibodies ($S/P < 1$). Diluted samples were then analysed by ELISA.

Matched milk and serum samples were analysed by regression analysis. Bulk milk titres from positive and 'negative' herds were compared using a Mann-Whitney rank sum test and the optimal cut-off bulk milk titre for differentiating positive and negative herds was calculated by plotting a receiver operating characteristic (ROC) curve.

Samples were stored at 4°C and analysed by ELISA within 4 weeks. Individual and bulk milk samples were collected into universals containing a preservative tablet (Broad Spectrum Microtabs II, D&F Control Systems, Dublin, California, US).

Results

1. Comparison of matched serum and milk samples

There was 95% agreement between serum and milk results when interpreted categorically (positive or negative) and the milk ELISA had a sensitivity of 90% and a specificity of 96% using the serum results as definitive. The correlation between the results for serum against matched milk samples is illustrated by the graph in figure 1.

2. Serial dilution analysis of antibody positive milk samples

The serial dilution analysis of positive milk samples in *Map* antibody negative milk followed what might be the expected pattern (see figure 2). Samples with lower initial S/P values fell into the negative range more quickly when diluted. As can be seen in the graph, the sample with a marginally positive titre (S/P=47) became negative (S/P<40) at a 1:2 dilution, whereas the sample with the highest initial titre (S/P=266) remained positive at a 1:32 dilution. Dropping the positive cut-off level to >10, which gave 100% specificity in the small population of herds in which bulk milk was tested (see below) led to the weakest sample becoming negative at a 1:8 dilution and the strongest sample remaining positive at the highest dilution of 1:128 (S/P=12).

3. Targeted cow and bulk milk sampling (Table 1)

Seventeen of the herds from which bulk milk samples were obtained were defined as being Johne's negative either because of known accreditation history (1 herd) or because their targeted individual samples gave no positive results (16 herds). The remaining 9 herds were defined as being Johne's positive because of known recent positive testing (3 herds) or because their targeted individual samples gave at least 1 positive result (6 herds).

There was a significant difference between the bulk milk titres from positive and designated negative herds (see figure 3, $p=0.004$; median values 11 and 4 respectively). The optimum cut-off point for differentiating these 2 groups was calculated as 9 (positive titre ≥ 9) by plotting an ROC curve. Using this cut-off point, bulk milk testing had a sensitivity of 0.67 (0.30, 0.93) and a specificity of 0.882 (0.64, 0.99) in detecting positive herds. All herds with bulk milk titres of greater than 10 were infected with Johne's disease.

Discussion

Quantifying antibodies to *Map* in individual milk samples is not currently included in the CHeCS rules as a valid method for herd screening. The findings in this study show that there should be no reason why individual milk samples cannot be used in the same

manner that they are permitted for use in IBR, BVD and leptospirosis testing. In our experience of cattle health scheme testing some farmers prefer this method of sampling. The goal of taking serum and milk samples from 6 targeted animals was not achieved in a number of herds. This may be of significance in designated negative herds as obviously, the fewer the number of samples tested, the less likely that a positive result will be found. Indeed, we recognise that any of the negative herds, apart from the accredited free herd, may have Johne's positive animals that were not detected by targeted screening. However, as the Johne's status of farms determined by targeted testing agreed very closely with farm history, we propose that targeted testing is a reasonably efficient method of detecting if the disease is a problem in a herd. Consequently, targeted testing could be of benefit to provide sources of replacement stock that has a 'likelihood of being free of disease'. Whilst by no means holding the status of whole herd screening, targeted testing is less of a financial burden and a need for this has been recognised by the dairy farming industry (Orpin 2005). Although 'proportional' sampling in Australia and the US use much larger numbers such as 30 or 50 animals tested, animals are not 'targeted' for testing in the same way that they were in this study (Wells 2002). Targeting suspect animals appears to allow lower numbers of animals to be tested, yet still produces useful results.

There were 3 farms in the study that had isolated cases of Johne's disease in the last 5 years in bought in animals. As infection is mainly considered to occur in young calves, it is likely that these animals were infected before they were bought. No positive cases were identified in these farms in the targeted samples. If the clinical cases were removed from the herd sufficiently early without contact with susceptible groups then it may be that there was no spread in the affected farms and the targeted samples gave a true picture of each farm's status. Alternatively, it may be that infection has spread on the farm, but is still at a pre-clinical stage and will only become apparent in time. Such is the conundrum of Johne's disease.

Bulk milk testing for antibodies to *Map* has not previously been recognised as being sensitive enough to detect herds with Johne's disease unless they are heavily infected (Nielsen 2000). Five out of the 9 positive herds had positive bulk milk titres when a lower positive cut-off level was used than for individual milks. This cut-off was selected so that all designated negative herds had negative bulk milk results (100% specificity), which was slightly higher than that determined by plotting a ROC curve. Using this cut-off level, the dilution series showed that the *Map* ELISA remained positive when a sample with an S/P of 266 was diluted 1:128 and when the sample with an S/P of 47 was diluted 1:4. Although it is tempting to extrapolate this to the potential detection of herds with a disease prevalence of <1% and 25% respectively, no assessment of herd prevalence of disease can be made in this study because of the lack of whole herd testing. In reality, herds with endemic Johne's disease will have a number of antibody positive cows with a range of titres. More of these animals will have titres at the lower end of the positive scale; as the titre increases, the animal is more likely to be culled from the herd due to the association with indirect and direct clinical effects. Further work is indicated to investigate the relationship between herd prevalence of disease, S/P levels of positive animals and bulk milk titres.

Conclusions

This study has three main conclusions. Firstly, individual milk samples give accurate results when quantifying antibodies to *Map*. Secondly, targeted sampling of 'high risk' animals can be of value in identifying herds with Johne's disease, although this approach may not always be accurate in herds with a low prevalence of disease. Thirdly, bulk milk sampling can also be used to identify herds with Johne's disease with the same proviso as targeted sampling. The results presented here support a rationale of using a lower cut-off point than that used for individual milk testing which would increase the sensitivity of the test. Many dairy farmers already regularly monitor bulk milk samples for antibodies to IBR, BVD and leptospirosis and there may be merit in including antibodies to *Map* in this assessment. These findings present the farmer and the dairy industry with cost-effective measures that may be of value in the assessment and control of Johne's disease.

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Figure 1 Graph of serum against milk ELISA results for matched samples. The broken lines correspond to the positive cut-off values for S/P in serum (≥ 70) and milk (≥ 40) on the appropriate axis. Disagreement between serum and milk results is shown by points in the upper left-hand and lower right-hand quadrants. The r^2 value for the line of best fit is 0.84 showing good correlation between both sets of results.

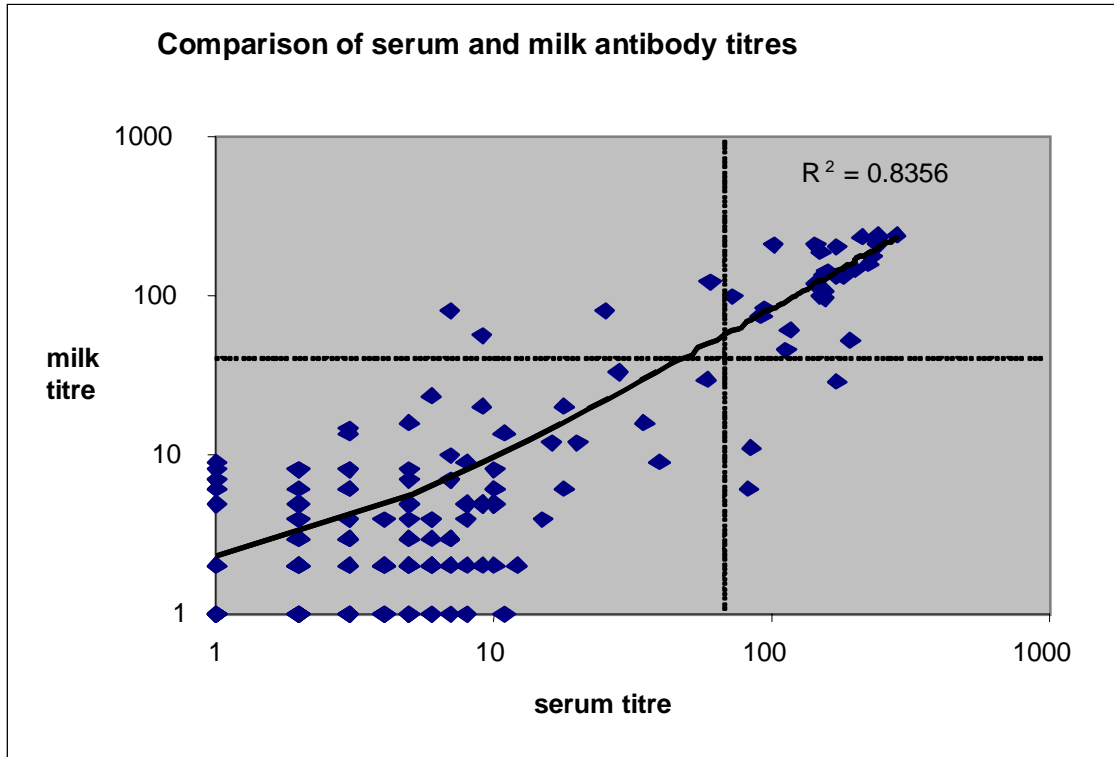


Table 1 Individual milk and serum results for the targeted animals tested and bulk milk titres for the herds involved.

Farm	Status determined from history	Targeted sampling results positive / no. tested		Bulk milk titre	Status determined from sampling
		serum	milk		
1	neg	0/6	0/6	<1	neg
2	neg	nt	0/6	1	neg
3	neg	0/3	0/6	1	neg
4	neg	nt	0/6	2	neg
5	neg	0/6	0/6	2	neg
6	neg	0/6	0/6	3	neg
7	neg	0/6	0/6	3	neg
8	neg	0/6	0/6	3	neg
9	neg	0/6	0/6	4	neg
10	neg	nt	0/6	5	neg
11	neg	nt	0/6	5	neg
12	neg	0/6	0/6	6	neg
13	neg	0/6	0/6	6	neg
14	neg	ht	ht	10	neg ^a
15	neg/pos ^b	0/6	0/5 ^c	5	neg
16	neg/pos ^b	0/6	0/6	6	neg
17	neg/pos ^b	0/6	0/6	10	neg
18	neg	1/6 ^d	1/6	5	pos
19	pos	ht	ht	4	pos ^e
20	pos	1/6	1/4	5	pos
21	pos	1/4	2/4	9	pos
22	pos	2/6	2/6	10	pos
23	pos	5/6	5/6	12	pos
24	pos	2/7	2/7	18	pos
25	pos	ht	ht	30	pos ^e
26	pos	ht	ht	99	pos ^e

nt = not tested ; ht = herd test

^a this herd had been 'accredited free' since 1997

^b history was of a single case of Johne's disease in a bought-in animal on these farms

^c 1 targeted cow was dry when tested

^d result recorded as positive fell in the doubtful category

^e multiple positive serology results at herd test

Figure 2: Graph of dilution series of positive milk samples against S/P value. Individual dilution curves of the 6 samples tested are shown on the graph. The dotted lines correspond to the positive level for individual milk samples (S/P = 40) and the cut-off in this study where all samples tested with titres above this were from positive herds (S/P =10).

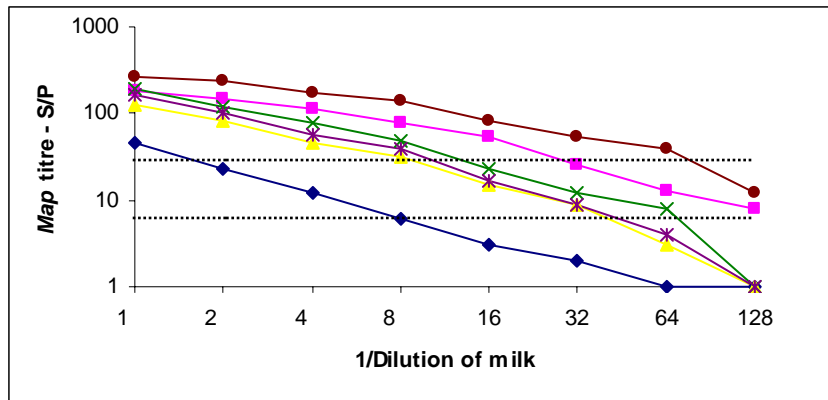


Figure 3: Graph of *Map* antibody titres in bulk milk samples for positive and negative herds. The median values in each group are shown by a horizontal line. The difference between the 2 groups is significant ($p=0.004$).

