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Scientific Article

Diagnosis of the Cu and Se status of dairy cattle in New Zealand: How many samples are needed?

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Abstract

AIM: To determine the minimum number of samples required to obtain a robust estimate of the Cu and Se status of dairy herds, as assessed by determining liver Cu and serum Se concentrations.

METHODS: Results were collated from analyses of samples of liver from 18 dairy herds and serum from 19 herds, for concentrations of Cu and Se, respectively. All herds were in either the Manawatu or Rangitikei regions of the North Island of New Zealand. Data were used to determine the required sample size for each herd; firstly to estimate the population mean with 90% confidence with a precision of 27.5 nmol/L for Se in serum, and 100 μmol/kg fresh weight (FW) for Cu in liver; and secondly to ensure that the 90% CI of the sample mean did not include specified thresholds for concentrations of Se or Cu.

RESULTS: For Se concentration in serum, the SD of each batch varied from 0.5–147 nmol/L, and for Cu concentration in liver, the SD varied from 173–829 μmol/kg FW. For Se, the minimum sample size required to estimate the population mean to within 27.5 nmol/L with 90% confidence was > 10 for 13/19 batches. For Cu, the minimum sample size required to estimate the population mean to within 100 μmol/kg FW was > 10 for 17/18 batches. When estimating required sample size based on 90% CI and a threshold value, the minimum sample size to confirm the population mean of Se was > 140 nmol/L was four in 17/18 batches where the sample mean was > 140 nmol/L. For concentrations of Cu in liver, ≥8 samples would have been sufficient for a threshold of 45 μmol/kg FW in 16/18 batches. For the 95 μmol/kg threshold, the minimum required was 12. For the threshold of 300 μmol/kg FW, 6/17 batches with a mean > 300 μmol/kg FW required ≥20 samples.

CONCLUSIONS: From this dataset of 21 herds, the sample size recommendation for ensuring that the population mean of Se concentration was not below the marginal threshold was similar to previous recommendations. For Cu concentrations in liver, the estimated sample size recommendations for ensuring that the population mean was not below the marginal threshold was much larger than currently recommended.

CLINICAL RELEVANCE: In dairy cattle, five to six blood samples per group should be taken to determine Se status, and to effectively monitor Cu status a minimum of 12 liver samples should be taken, preferably in the autumn.

KEY WORDS: *Dairy cows, liver Cu, serum Se, deficiency, sample size*

Introduction

Sampling animal tissues is a commonly used and effective method of diagnosing and preventing mineral deficiency (Underwood and Suttle 1999). When such sampling is undertaken, the number of samples required is a key criterion. If too many animals are sampled then costs will be higher than they need to be and animals will have been sampled unnecessarily, but if too few animals are sampled then no conclusions can be drawn because estimates of prevalence or means will have confidence intervals that are too large and the exercise will have been costly and futile (Grace *et al.* 2010). Despite this, sample size is often not considered appropriately when investigations of trace element status are planned, with sample numbers being based on cost and practicality (Fraser 1982), on the numbers tested on other farms or, at best, on laboratory-produced tables (e.g. Fraser 1982; Clark and Ellison 1993), rather than being based on farm-specific data. Furthermore, once the data are received from the laboratory, post-hoc calculations to confirm that the correct number of samples were taken is not a routine procedure, so valuable information that could guide further testing is lost or ignored.

In herds with more than 100 animals, in which herd size has only a small impact on the sample size required (Fraser 1982), the required sample size depends principally on three factors (Dohoo *et al.* 2003). Firstly, how precise the estimate of population mean needs to be, i.e. how big a difference can be allowed between the estimate (sample mean) and the actual (population) mean. The more precise the estimate needs to be, the more samples are needed, e.g. to be within 1 μmol/kg of the actual mean, more samples are required than to be within 10 μmol/kg. For mineral status evaluation, the precision of the estimate is often taken to equal half of the marginal range (Fraser 1982), i.e. half the difference between the marginal and deficient thresholds.

The second factor is the confidence level of the estimate of the population mean. A confidence interval is a range over which the

FW Fresh weight

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data suggest that the true population mean will lie. If the mean is 4 nmol/L and the 90% CI is 3–5 nmol/L we can be 90% confident that the population mean lies within that range. A 95% CI is often used in research (Dohoo *et al.* 2003) but a lower figure of 90% is often used when evaluating mineral status (Fraser 1982). The third factor is the expected variability of the data, i.e. the variation between results from individual animals. Ideally, the within-herd variance should be used to calculate sample size, but this is often not known or, in herds where samples are taken routinely, not calculated. The absence of this statistic is probably the key reason why sample size calculations are not undertaken for most mineral status investigations.

For assessing mineral status, another key criterion is whether the outcome of interest is the mean of concentrations in a tissue or the proportion of animals with concentrations less than the deficient or marginal threshold, as the formula for the calculation of sample size depends on whether the mean or the proportion is the crucial variable (Dohoo *et al.* 2003). In New Zealand, the mean is usually the basis for interpretation, although individual results may be used, particularly in supplemented herds (Fraser 1982; Clark and Ellison 1993).

In a brief report, Fraser (1982) stated that measurement of Se in whole blood required three samples in order to be able to identify the mean to within half of the marginal range (i.e. within 65 nmol/L) with 90% confidence. For Cu, using the same criterion (with half the marginal range being 25 μmol/kg fresh weight (FW)), that author concluded that 16 samples were required, but he also stated that if the precision of the estimate was increased to the whole marginal range (50 nmol/kg) the sample size could be reduced to four. These recommendations regarding sample size have remained largely unchanged. However in subsequent correspondence, Clark and Ellison (1993) recommended that in herds supplemented with Se, sample size should be increased to 10, due to the increased variability in concentrations of Se within such herds, and because increasing sample size allowed individual concentrations to be taken into account. Those authors also recommended the use of serum for measurement of Se in such situations, rather than glutathione peroxidase activity, because Se in serum more rapidly reflected the status of the animal after supplementation.

For the assessment of Cu concentrations in liver Clark and Ellison (1993) also recommended at least four samples, but Grace *et al.* (2010) suggested that the minimum number of samples should be increased to 12 because of high within herd variability. They reported within-herd SD of between 291–766 μmol/kg FW, much higher than the 60 μmol/kg FW used by Fraser (1982). Grace *et al.* (2010) based their sample size recommendation on a pragmatic maximum for the confidence interval of the sample mean of 425 μmol/kg FW. Therefore in their dataset even the increase to 12 samples per group resulted in markedly less accuracy than was reported with four samples by Fraser (1982). However, all the data obtained by Grace *et al.* (2010) came from the Waikato region and it is possible that the within herd variability observed in this region was unusual. So further data from elsewhere in New Zealand are required before recommendations can be made for increasing sample numbers for measurement of Cu concentrations in liver.

Additionally, since the earlier sample size calculations were reported by Fraser (1982) and Clark and Ellison (1983), the marginal range for serum Se concentration has been properly characterised as 85–140 nmol/L (Thompson *et al.* 1998). This range of 55 nmol/L, is smaller than the marginal range, of 120 nmol/L for whole blood, used by Fraser (1982). For Cu there has been no major changes in the marginal range, except for those that resulted from changing to SI units. This range is from 45 to 95 μmol/kg FW, so is 50 μmol/kg wide (Balemi *et al.* 2010). It has also been shown that in sheep and beef cattle in New Zealand Cu concentrations in liver decrease significantly during the winter. So Ellison (1994) recommended that mean Cu concentrations in the liver of cattle in the autumn should be >300 μmol/kg FW to ensure that they did not become marginal (i.e. <95 μmol/kg FW) before calving. This is now the standard threshold recommended when Cu concentration in liver is measured in dairy cattle in the autumn, rather than either the marginal or deficient thresholds (Grace *et al.* 2010).

These changes in size of the marginal range (for Se) and variability and threshold (for Cu) suggest that we need a reassessment of sample size requirements for assessing the concentration of Cu in the liver and Se in serum. The aim of this analysis was to assess whether the criteria used by Fraser (1982) and Clark and Ellison (1983) to estimate required sample size still produced minimum sample size estimates that were robust, and, if not, whether taking into account diagnostic thresholds reduced those sample size estimates.

Materials and methods

Data were collected from the results of analyses of samples from 22 visits to 21 dairy herds between April 2010 and April 2011, where either liver biopsy was used to assess Cu concentrations or serum was taken to assess Se status. All visits were made by private veterinarians as part of mineral management programmes for the herds. Four visits just involved sampling for Se concentrations, two just for Cu concentrations, and 16 visits involved both. All farms were in either the Manawatu or Rangitikei regions of the southern part of the North Island of New Zealand. All liver samples were taken during autumn (April /May). All herds routinely used oral Cu supplementation and except for two herds which were supplemented with organic Cu year round, this supplementation had ceased in January when supplementation with zinc to prevent facial eczema had started (Dawson and Laven 2007). Se supplementation was used in all but two herds with most ceasing in January.

All samples were analysed by a commercial laboratory (New Zealand Veterinary Pathology, Hamilton, NZ). Cu concentrations in liver were assessed using nitric/perchloric acid digestion, followed by flame atomic absorption spectrometry, and Se concentrations in serum were assessed using atomic absorption spectrometry.

Sample size estimation based on the precision of the estimate of the population mean

This analysis calculated the minimum number of samples required in order to estimate the population mean to a predefined level of precision, as used by Fraser (1982). For Se a minimum precision of 27.5 nmol/L was used to estimate sample size, while for Cu a minimum precision of 100 μmol/kg FW was used (Laven 2011). Cu and Se sample size data were both calculated using 90% CI. All sample size calculations were undertaken using the sampling menu of StatPac (StatPac Inc.

Minnesota MN, USA). No account of mean value was used in this analysis.

For Se data, the equation used to calculate required minimum sample size was: sample SD $*$ $1.65^2/27.5^2$

For the Cu data the equation was the same, except that 27.5 was replaced by 100.

Sample size estimation based on whether the 90% confidence interval included a diagnostic threshold

For this analysis the difference between the sample mean and the diagnostic threshold was calculated, as was the sample SD of the sample population. Both were then used to estimate the minimum number of samples (n) required to ensure that the 90% CI of the sample mean did not include that threshold, i.e. we were 90% confident that the true population mean was not lower than our chosen threshold, assuming that increasing sample number would not have altered sample mean or SD.

The formula used to calculate the confidence interval was: t $*$ sample SD/$\sqrt{}$(n)

Where t is the critical value for the t-distribution at the 90% confidence level with n-1 degrees of freedom (Dohoo *et al.* 2003).

All analyses were undertaken using SPSS 17.0 (SPSS Inc. Chicago IL, USA). The thresholds used were the marginal threshold for Se of 140 nmol/L (Thompson *et al.* 1998), the deficient threshold for Cu of 45 μmol/kg FW, the marginal threshold for Cu of 95 μmol/kg FW (Balemi *et al.* 2010) and the 300 μmol/kg FW threshold suggested by Ellison (1994) as being sufficient liver Cu reserves for in-calf dairy cattle at the onset of winter.

Table 1. Number of samples collected from 19 herds of dairy cows, with the mean and SD concentration of Se in serum (nmol/L) and estimated minimum sample size. Marginal range for Se in serum: 85–140 nmol/L (Thompson *et al.* 1998).

Sample batch ID	Number of samples	Mean Se in serum	SD	Estimated minimum sample size[a]
21	5	50	0.5	1
2	5	158	11	1
22	6	175	79	22
10	7	193	38	5
9	6	262	90	28
8	5	364	105	39
11	5	376	95	32
5	10	412	143	73
12	5	628	123	54
15	6	637	147	77
6	5	678	43	6
4	4	687	84	25
1	4	722	42	6
14	6	730	66	15
18	11	734	79	22
19	5	788	97	33
13	7	790	94	32
3	4	795	70	17
17	5	904	47	7
Median		637	79	22

[a] Calculated based on ensuring that the population mean is estimated to within half of the marginal range (i.e. 27.5 nmol/L) with 90% confidence

Results

The results of the sample size calculation are summarised for Se in Table 1 and for Cu in Table 2. Of the 22 batches of samples all but one had a mean Se concentration in serum >140 nmol/L, and all batches had a mean Cu concentration in liver >95 μmol/kg FW. Using the threshold of 300 μmol/kg FW for sufficient reserves of Cu, 1/18 batches had a mean Cu concentration in liver less than this, whereas if 500 μmol/kg FW was used, as suggested by Grace *et al.* (2010), then 7/18 batches had results indicating insufficient liver Cu reserves.

Sample size estimation based on the precision of the estimate of the population mean

For Se, batch SD varied from 0.5–147 nmol /L (Table 1). Of the 19 batches only five had a SD <68 nmol/L, the SD used by Fraser (1982), although the median SD was 79 nmol/L. The marginal range for Se in serum is smaller than that for whole blood, and 17/19 batches had an estimated sample size requirement >6, i.e. at least twice that suggested by Fraser (1982), and in 13/19 cases >10, as suggested by Clark and Ellison (1993).

For Cu, the SD of each batch varied from 173–829 μmol/kg (Table 2); in all cases this was markedly more than the 60 μmol/kg FW used by Fraser (1982). Therefore the required precision of the estimate (within 100 μmol/kg FW) was much less than the 50 μmol/kg FW used by Fraser (1982) to calculate a requirement of a minimum of four liver samples for measurement of Cu in liver. Nevertheless, only one batch of samples from this study had an estimated minimum sample size <10.

Sample size estimation based on whether the 90% CI included a diagnostic threshold

Analysis of the Se data showed that only one batch (batch 22) had a sample mean concentration of Se >140 nmol/L but had a lower 90% CI that included the marginal threshold (<140 nmol/L), indicating that too few samples had been taken to confirm adequate status. In that case, 13 samples would have been required to prevent such overlap. For all other batches, four samples would have been sufficient to confirm adequate status.

For the Cu data, despite none of the batches having a mean concentration of Cu in liver <160 μmol/kg FW, five batches had 90% CI of the population mean that included 95 μmol/kg FW (the upper limit of the marginal range), three of which (batches 15, 6 and 22) had 90% CI that included the lower limit of the marginal range (45 μmol/kg FW).

In this dataset, five batches would have required ≥ 6 samples to have 90% CI that did not include the threshold for concentrations of Cu in liver of 45 μmol/kg FW; this increased to six batches when the threshold was increased to 95 μmol/kg FW. In order to identify, in approximately 90% of batches (i.e. 16/18), that the population mean was >45 μmol/kg FW (with 90% confidence) would have required ≥ 8 samples per batch. For a 95 μmol/kg threshold, the equivalent figure would have been 12 samples. If only four samples had been taken, 7/18 batches would not have had a mean significantly >45 μmol/kg FW (with 90% confidence), and 9/18 would not have had a mean significantly >95 μmol/kg FW.

Table 2. Number of samples collected from 18 herds of dairy cows, with the mean and SD concentration of Cu in liver (μmol/kg fresh weight (FW) and estimated minimum sample size. Marginal range for Cu in liver: 45–95 μmol/kg FW (Lee *et al.* 2002).

Sample batch ID	Number of samples	Mean liver Cu	SD	Estimated minimum sample size[a]
6	5	163	173	8
22	6	350	404	44
15	6	389	562	85
20	8	401	694	130
5	8	425	418	47
2	5	429	354	33
4	5	437	347	32
9	6	506	354	33
16	6	506	339	31
18	11	606	466	58
10	8	708	379	38
17	5	898	382	38
3	5	1074	648	113
7	4	1207	719	139
1	4	1420	732	144
11	5	1428	762	157
12	5	1860	350	33
13	9	2591	829	185
Median		556	411	45.5

[a] Calculated based on ensuring that the population mean is estimated to within twice the marginal range (i.e. 100 μmol/kg FW) with 90% confidence

For a threshold for concentrations of Cu in liver of 300 μmol/kg FW, 6/17 batches with a mean >300 μmol/kg FW would have required ≥20 samples to confirm that their actual mean was >300 μmol/kg FW. All of these batches had a mean Cu concentration in liver of <440 μmol/kg FW; for the three batches with a mean between 500–700 μmol/kg FW, nine or 10 samples would have been required to ensure that the population mean was not <300 μmol/kg FW.

Discussion

Copper and Se deficiency have both been well documented in dairy herds in New Zealand (e.g. Smith and Coup 1973; Wichtel 1998). Therefore dairy herd animal health programmes should include an assessment of Cu and Se status so that deficiency can be prevented by implementing, if necessary, supplementation of Cu and/or Se. There is a significant seasonal trend in Cu concentrations in liver in cattle in New Zealand – West and Sargison (1998) reported that there was a three-to-four fold decline between autumn (when liver Cu concentration peaked) and spring – so the best time to evaluate Cu concentrations in liver is in the autumn. For Se, there is no such seasonal trend but sampling at the same time has logistical benefits.

The aim of this study was to assess whether current recommendations on sample size for Cu and Se status provide a robust estimate of Cu or Se status. The analysis was based on results from 21 farms in one region of New Zealand and was mostly undertaken on farms where Cu and Se status was expected to be adequate, either because of supplementation or based on farm history. The applicability of these results to other farms with different histories may be limited. Further results from around New Zealand from herds with different histories, production levels, feeding regimes and supplementation strategies are needed to confirm these findings. Nevertheless, these tests are typical of results where animals are sampled in order to confirm that mineral status is adequate, so this analysis does highlight the problem of interpreting the results of such testing.

Ideally, to identify status all animals would be tested and the herd mean identified exactly. However this is impractical and costly, so a proportion of the herd is tested. If too few samples are taken this sample mean may not accurately reflect the population mean, particularly if the variability is high. The present dataset showed that for both Se and Cu there was large variation between and within herds; this is likely to be a reflection of the level of supplementation in these herds. This variability meant that for Se, simply aiming to estimate the herd mean Se concentration to within half of the marginal range was not feasible. However, taking into account the sample mean, which meant that the precision of the estimate could be reduced, allowed the required sample size to be reduced to a minimum of four, confirming that in all batches sufficient samples had been taken originally. As a result, simple interpretation of the sample means would have accurately identified that one of the 19 batches tested was deficient (<50 nmol/L; batch 21), one was near marginal (batch 22) and the remainder were adequate, with a population mean significantly >140 nmol/L (Table 1). The conclusion that 5–6 samples is sufficient to diagnose Se status is consistent with published studies on the Se nutrition of cattle that have used 5–6 animals per treatment to determine changes in blood and serum Se concentrations in Se deficient cows, and the impact that various Se supplements had on Se status (e.g. Thompson *et al.* 1981; Fraser *et al.* 1987; Wichtel *et al.* 1996).

For liver Cu, taking account of the sample mean resulted in a minimum sample size estimate of 12 samples per group, substantially greater than that recommended by either Fraser (1982) or Clark and Ellison (1993), but the same as that recommended by Grace *et al.* (2010). Only one of the 18 tested batches had a number of samples close to this maximum; so although all the sample mean concentrations of Cu were greater than 160 μmol/kg FW (which if true would indicate that all of the herds had adequate Cu status), five of the 18 tested herds had a 90% CI of the population mean that included 95 μmol/kg FW: i.e. although the sample mean was in the adequate range, the population mean had >10% chance of being in the marginal range.

The recommended figure of 12 liver samples is relatively high, so there may be some resistance to this change. One statistical alternative that is often suggested is to use the proportion of individual values below a threshold. For example, supplementation could be recommended when >10% of the herd have Cu concentrations in liver <95 μmol/kg FW. However, to show, with 90% confidence, that <10% of the herd have such a concentration would require 21 negative samples (Dohoo *et al.* 2003).

The recommendation of 12 samples per herd is also supported by published studies on Cu nutrition of cattle, both field and experimental, where similar numbers of cattle have been tested (e.g. Spolders *et al.* 2008; Balemi *et al.* 2010; Hittmann *et al.* 2012; Scaletti and Harmon 2012). These studies, taken together with the findings from the present study, strongly support the

recommendation by Grace *et al.* (2010) that the Cu status of grazing dairy cows in New Zealand can be assessed from 10–12 animals per herd, sourcing liver either via biopsy or via collection at the time of slaughter.

In conclusion, assessing the Cu and Se status of dairy cattle to ensure that animals do not become deficient should be a key part of the nutritional management of animal health. The present analysis, supported by data from other published studies, suggests that blood samples from 5–6 animals per herd should be sufficient to determine Se status, and liver samples from 10–12 cattle per herd should be sufficient to determine Cu status.

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