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## Relationship between blood phylloerythrin concentration and gamma-glutamyltransferase activity in facial eczema-affected cattle and sheep

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### ABSTRACT

Phylloerythrin (PLE) is a photo-dynamic compound, a natural breakdown product of chlorophyll. Its concentration and relationship with gamma-glutamyltransferase (GGT) were determined in animals seriously affected with facial eczema (FE). Seven commercial cattle herds ( $n = 1,455$ ) and one experimental sheep flock ( $n = 597$ ), each containing clinically affected animals, were monitored. On a pooled within-farm basis, mean PLE concentration was significantly higher in clinical FE cases than in non-clinical animals in cattle (28.7 vs 16.0 relative units,  $P < 0.001$ ), and in lambs (0.282 vs 0.024  $\mu\text{g/ml}$ , respectively,  $P < 0.001$ ). PLE concentration rose in some, but not all, animals when GGT activity exceeded  $\sim 600$  IU/L in cattle and  $\sim 400$  IU/L in lambs (Reference GGT range in normal (unchallenged) animals: 0 to 40 IU/L, cattle; 32 to 70 IU/L, sheep). A heritability for PLE concentration was estimated in cattle ( $0.19 \pm 0.07$ ), and phenotypic and genetic correlations with  $\text{Log}_e\text{GGT}$  were  $0.37 \pm 0.02$  and  $0.92 \pm 0.09$ , respectively. In the lambs the phenotypic correlation between  $\text{Log}_e\text{GGT}$  and PLE was  $0.32 \pm 0.04$ . Thus PLE is a partially inherited trait, and is associated with visible signs of FE, especially above GGT thresholds of  $\sim 600$  IU/L in cattle and  $\sim 400$  IU/L in lambs.

**Keywords:** facial eczema; cattle; sheep; gamma-glutamyltransferase; phylloerythrin; photosensitivity.

### INTRODUCTION

Phylloerythrin (PLE), or phytoporphyrin, belongs to a class of natural pigments known as porphyrins. It is a breakdown product of chlorophyll, which is abundant in the diet of grazing ruminants, through microbial fermentation in the rumen (Quin *et al.*, 1935). The intact chlorophyll molecule is too large to be absorbed into the bloodstream (Johnson, 1983), whereas the PLE molecule is smaller. Most PLE passes through the gastro-intestinal tract and is excreted in the faeces, but some is absorbed and enters the hepatic portal circulation (Tennant, 1998), from where it eventually enters the bile duct and gall bladder for excretion.

In cases of liver and bile duct injury, such as occurs commonly in cattle and sheep after ingesting sporidesmin, the fungal toxin causing facial eczema (FE), the absorbed PLE spills over into the systemic circulation (Tennant, 1998). PLE is a photo-dynamic pigment, which causes damage to cells in unpigmented or lightly pigmented areas of the skin when exposed to sunlight. The resulting photosensitivity has been observed within as little as 40 minutes after experimental intravenous administration of PLE to lambs (Clare 1944). He reported that "at the end of three hours the ears were somewhat swollen and sensitive when touched". Skin lesions begin "as an immediate erythema, followed soon afterwards by oedema.....[and later] some oozing of serum, scab formation and loss of hair" (Smith & O'Hara, 1978).

In time-series data from ruminants receiving oral sporidesmin as an artificial challenge, glutamate dehydrogenase enzyme production, as an indicator of hepatocyte damage, precedes gamma-glutamyltransferase (GGT) enzyme production (Morris *et al.*, 1998). GGT is an indicator of bile duct damage. In turn GGT production precedes PLE production (Scheie *et al.*, 2003b). Our objective in this report is to explore the relationships between blood PLE concentration and GGT activity, in cattle and sheep clinically affected with FE.

### MATERIALS AND METHODS

#### Ethics

This work was carried out with the approval of the relevant Animal Ethics Committees, at AgResearch Ruakura and AgResearch Invermay.

#### Animals sampled

##### Cattle

The herds in this study were part of a larger Ruakura project on FE resistance in cattle (Cullen *et al.*, 2006). Criteria for sampling each herd were: grazing groups contained at least 3% of animals the herd-owner deemed to be clinically affected with FE after natural challenge on a mixed ryegrass/white clover diet; at least 30% of animals had elevated GGT levels ( $>40$  IU/L). In all qualifying herds, a blood sample was obtained in a 10 mL heparinised vacutainer from all animals in the grazing group at one time, regardless of the stage of progression of the disease. Plasma GGT enzyme activity was then

**TABLE 1:** Effect of observed clinical status on least square means for gamma-glutamyltransferase (GGT) activity and phylloerythrin (PLE) concentration, in plasma samples from cattle in herds affected by facial eczema.

Parameter	Herd 1	Herd 2	Herd 3	All Herds
Number of animals sampled	294	257	88	639
Number of clinical cases	15	12	6	33
Percent clinical cases	5.1%	4.7%	6.8%	5.2%
GGT (IU/L)				
Range	5 – 3,890	7 – 3,916	45 – 3,067	
Log <sub>e</sub> GGT least squares mean	7.03	6.75	6.76	
Mean GGT (back-transformed)	1129	855	866	
Difference <sup>a</sup> ± SE (Log units)	1.70 ± 0.40	2.13 ± 0.44	0.38 ± 0.63	1.40 ± 0.29
Significance	<0.001	<0.001	NS	<0.001
PLE (relative units)				
Range	9.3 - 57.0	7.8 - 33.0	9.7 - 54.7	
Mean	24.1	21.9	21.1	
Difference <sup>a</sup> ± SE	16.8 ± 1.8	12.9 ± 2.0	8.4 ± 2.8	12.7 ± 1.3
Significance	<0.001	<0.001	<0.003	<0.001

<sup>a</sup> Value in the clinical group minus value in the non-clinical group ± standard error (SE).

determined, and DNA extracted from the white blood cells. Additionally, in autumn 2007, plasma PLE concentration was determined from samples in seven of these qualifying herds. Six were mixed-age dairy cows and one was weaned beef calves. A total of 1,455 cattle were sampled. Information on the clinical FE status of individual animals was available from three herds with the details shown in Table 1.

**Sheep**

The sheep study was carried out at AgResearch’s Woodlands Farm near Invercargill, an FE-free region. In order to study genetic variation in experimentally-induced FE susceptibility, a lamb flock was generated by mating three crossbred Finn x Texel sires to Coopworth ewes. The resulting lambs were weaned at approximately 3 months of age and thereafter grazed together on mixed ryegrass/white clover pasture. A serum blood sample was obtained from all lambs before dosing with sporidesmin, to check that GGT activity was within the normal range for unchallenged animals of 32 to 70 IU/L. At an average of five months of age, all lambs received a single oral dose of sporidesmin at a rate of 0.3 mg/kg live weight. Weekly serum samples were obtained from all lambs for five weeks thereafter, and the GGT and PLE data in the present analyses were obtained from results for the samples taken in the third week. All animals were monitored carefully for clinical signs of FE. Affected animals were recorded and given access to shade, feed and water.

**Laboratory analyses**

Blood samples were analysed immediately after collection for GGT activity at 37°C on a Hitachi-

Roche modular autoanalyser using the method of Persijn and Van der Slik (1976), and standardised according to International Federation of Clinical Chemistry methods (Shaw *et al.*, 1983). Samples were stored at -20°C for a period of at least a month before PLE analyses were carried out. Duplicate 200-250 µL samples were assayed for PLE

**TABLE 2:** Effect of observed clinical status on least square means for gamma-glutamyltransferase (GGT) activity and phylloerythrin (PLE) concentration in serum samples from lambs artificially challenged with sporidesmin.

Parameter	Flock 1
Number of animals sampled	597
Number of clinical cases	85
Percent clinical cases	14.2%
GGT (IU/l)	
Range	34 – 1,185
Log <sub>e</sub> GGT least squares mean	4.90
Mean GGT (back-transformed)	134
Difference <sup>1</sup> ± SE (Log units)	1.87 ± 0.09
Significance	<0.001
PLE (µg/ml)	
Range	0.005 – 2.00
Mean	0.061 <sup>b</sup>
Difference <sup>1,2</sup> ± SE	0.258 ± 0.021
Significance	<0.001

<sup>1</sup> Value in the clinical group minus value in the non-clinical group ± standard error (SE).

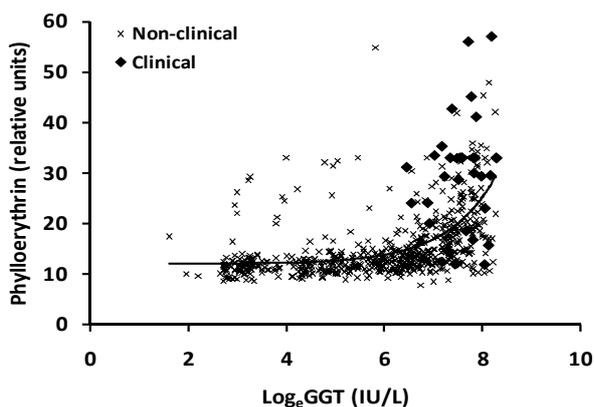
<sup>2</sup> An alternative analysis, using Log<sub>e</sub>(PLEx100), gave a raw mean of 1.04 units, and a difference between clinical and non-clinical cases of 1.88 ± 0.06 units (P <0.001).

concentration at 650 nm (band width 10 nm) by the spectrofluorometric method of Scheie *et al.* (2003a) at 420 nm excitation (band width also 10 nm), using a BMG LABTECH Fluorescence Plate Reader (Model FLUOstar OPTIMA). The cattle plasma samples were measured directly for PLE fluorescence intensity relative to a PLE-free plasma control. There was no standard available at the time. For the sheep serum samples, PLE (Porphyrin Products, Logan, USA) was dissolved in absolute methanol to a concentration of 0.1 mg/mL as a stock solution and stored at -20°C. On the day when the fluorescence emission and excitation spectra were measured, the PLE stock solution was further diluted to a concentration of 0.001 mg/mL. Incremental amounts of this solution were added to serum from clinically normal sheep displaying no visible signs of FE, to make standards containing varying PLE concentrations, for a standard curve. The weak stock solution was used in order to circumvent a precipitation problem. Fluorescence of the experimental samples was recorded against a standard curve at the final dilution (0-0.001 mg/mL), and values for the control samples were subtracted. PLE concentrations for each sheep are given as the average of 10 readings from each duplicate.

#### Statistical analyses

The GGT data were transformed to natural logarithms before analysis, because of the extended distribution for the high values. Activities of the GGT enzyme and concentrations of PLE were analysed using least squares in the SAS JMP (SAS, 1995) package, adjusting for herd and age in the

**FIGURE 1:** Relationship between  $\text{Log}_e$ GGT enzyme activity and plasma phylloerythrin (relative fluorescence intensity) in cattle from three herds unintentionally exposed to sporidesmin.  $\text{Log}_e$ GGT value of 3.69 corresponds with a GGT value of 40 IU/L (upper bound for unaffected animals);  $\text{Log}_e$ GGT values of 6.4 or 6.9 correspond with GGT values of about 600 and 1,000 IU/L.



case of the cattle, and testing for effects of observed animal status as clinical FE *versus* non-clinical, on a within-herd/flock basis. Animal-model restricted maximum likelihood analyses were also used in the cattle data (Gilmour, 1997), with a repeated-record model, to estimate heritabilities for  $\text{Log}_e$ GGT and PLE, and the genetic correlation between them. In the animal model, contemporary group was fitted as a fixed effect, consisting of herd x grazing group x year combinations.  $\text{Log}_e$ GGT values were standardised within this fixed effect. There were only three sires in the trial design for the experimental sheep, so there was no opportunity to estimate genetic parameters in the same way as with the cattle.

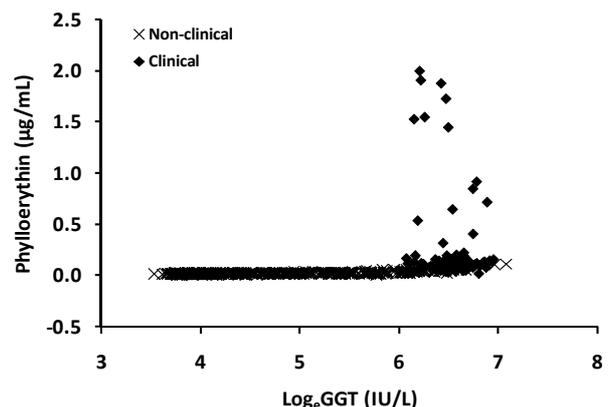
## RESULTS

### Cattle

Table 1 shows the herd means and effects of observed animal status on GGT activity and PLE concentration. In two of the three herds, the clinical *versus* non-clinical animal effects on least squares means for  $\text{Log}_e$ GGT and PLE differed significantly within herd. Clinical status was significant for both traits when all herds were combined ( $P < 0.001$ ). Between the clinical and non-clinical groups,  $\text{Log}_e$ GGT activities differed overall by  $1.40 \pm 0.29$   $\text{Log}_e$  IU/L, and relative PLE concentrations differed overall by  $12.7 \pm 1.3$ . The curvilinear relationship between  $\text{Log}_e$ GGT and PLE is shown in Figure 1, with a point of inflection for  $\text{Log}_e$ GGT at  $\sim 6.4$   $\text{Log}_e$  units, which is equivalent to a GGT value of  $\sim 600$  IU/L. Not all animals with GGT values above this threshold were clinical cases, but none of the clinical cases had GGT values below the threshold.

The heritability estimate for standardised  $\text{Log}_e$ GGT, from all data collected up to October 2008, was  $0.36 \pm 0.03$ . The estimate for PLE

**FIGURE 2:** Relationship between  $\text{Log}_e$ GGT enzyme activity and serum phylloerythrin concentration in lambs, three weeks after oral dosing with sporidesmin.



concentration, from the much more limited data available of six herds with 1,367 records where pedigree was also available, was  $0.19 \pm 0.07$ . Phenotypic and genetic correlations between the two traits were  $0.37 \pm 0.02$  and  $0.92 \pm 0.09$ , respectively.

### Sheep

Results for the experimental sheep flock are summarised in Table 2 showing that, three weeks after sporidesmin dosing, the mean  $\text{Log}_e\text{GGT}$  was 4.90 (back-transformed value = 134 IU/L), and there was a highly significant difference of  $1.87 \pm 0.09$  Log units ( $P < 0.001$ ) in  $\text{Log}_e\text{GGT}$  value between clinical and non-clinical cases. This difference was equivalent to a factor of 6.5 between GGT activity levels of the two groups. PLE concentrations of the clinical *versus* non-clinical cases also differed significantly ( $P < 0.001$ ), and there was a factor of 11.8 (0.282: 0.024  $\mu\text{g}/\text{mL}$ ) between the two least squares means. The phenotypic correlation between  $\text{Log}_e\text{GGT}$  and PLE concentration was  $0.32 \pm 0.04$ . A curvilinear relationship between  $\text{Log}_e\text{GGT}$  and PLE was observed (Figure 2), as with the cattle, with a point of inflection for  $\text{Log}_e\text{GGT}$  at  $\sim 6.0$  Log<sub>e</sub> units, equivalent to a GGT value of  $\sim 400$  IU/L. The threshold value may be specific to this flock and year.

## DISCUSSION

In general our cattle and sheep results indicated that only those with high GGT values, that is animals with severe liver damage, became clinically photosensitive. In the cattle group the GGT threshold was approximately  $>600$  IU/L and in sheep it was  $>400$  IU/L. As expected, the photosensitive animals generally showed higher PLE concentrations in the blood.

We found many animals showing anomalous phenotypes with respect to GGT levels, PLE concentrations and photosensitivity. Taking the experimental sheep flock as an illustration of this phenotypic variation, there were 459 non-clinical animals with GGT values below 400 IU/L (average GGT 107 IU/L, range 34 to 392 IU/L), and average PLE value of 0.021  $\mu\text{g}/\text{mL}$  (range 0.005 to 0.061  $\mu\text{g}/\text{mL}$ ). The remaining 138 animals consisted of 85 clinical cases (average GGT of 694 IU/L (range 402 to 1,043 IU/L), and average PLE of 0.285  $\mu\text{g}/\text{mL}$  (range 0.021 to 2.0  $\mu\text{g}/\text{mL}$ )), and 53 non-clinical cases (average GGT 559 IU/L (range 404 to 1,185 IU/L), and average PLE of 0.050  $\mu\text{g}/\text{mL}$  (range 0.022 to 0.131  $\mu\text{g}/\text{mL}$ )). This indicates that although animals may have suffered similar liver damage, they did not all show elevated PLE concentration and photosensitivity. A simplistic extrapolation is that some animals may have livers capable of removing more PLE than others (Smith & O'Hara, 1978).

Heritabilities for  $\text{Log}_e\text{GGT}$  and PLE in the cattle data were  $0.36 \pm 0.03$  and  $0.19 \pm 0.07$ , respectively. The  $\text{Log}_e\text{GGT}$  value had a lower standard error because of many more records. Both were the result of a sampling regimen using animals naturally exposed to sporidesmin. Hence variation in pasture intake, and thus toxin, was not controlled. Animal records in the data were processed taking account of the physical contemporary groups in which the animals were grazing, and also their age to adjust, as far as possible, for previous years of exposure.

There are possibly many factors influencing the levels of PLE concentration in the blood. In early sheep studies in South Africa, Quin *et al.* (1935) observed between-animal variation in faecal PLE output, for animals on the same diet and with similar feed intakes. There may also be between-animal variation in the host's ability to control microbial fermentation rates in the rumen, affecting hydrolysis of chlorophyll to PLE. Host-controlled fermentation differences are implied, for example, by heritabilities of ketone concentrations in milk or blood from postpartum cows (Gravert *et al.*, 1991; Tveit *et al.*, 1992, respectively), by between-cow differences in methane emissions under controlled grazing conditions (Pinares-Patiño *et al.*, 2003), and by metabolic differences associated with residual feed intake in steers (Richardson *et al.*, 2004). Together these would be consistent with heritable differences in PLE concentration in cattle, as found in this study.

The amount of assimilated PLE that then spills over from the bile duct is determined by the degree of bile duct injury. Since it is the PLE that leads to the external clinical signs of FE, it was expected that clinical cases would show higher concentrations of PLE than non-clinical cases. This was demonstrated for both cattle and sheep in Figures 1 and 2. In both species, a positive phenotypic correlation was observed between  $\text{Log}_e\text{GGT}$  and PLE concentration of 0.37 in cattle, 0.32 in sheep. The clinical cases, with a higher PLE, generally had higher  $\text{Log}_e\text{GGT}$  values. The reverse was, however, not necessarily true in that the non-clinical cases did not necessarily have a low GGT value. A candidate gene involved with retaining the integrity of bile duct cells is the ATP-binding cassette transporter G2 (*ABCG2*) (Morisaki *et al.*, 2005). It has been shown that the gene is involved in sensitivity to FE in sheep (Duncan *et al.*, 2007), and that PLE is a substrate of the gene (Robey *et al.*, 2006).

In summary, points where the host's genetic variation in PLE could be expressed include:

- Control of microbial fermentation rates, including hydrolysis of chlorophyll to PLE.
- Variation in the proportion of PLE channelled to the hepatic portal system indicating

selective absorption from the gut, as suggested from the study by Quin *et al.* (1935).

- *ABCG2* or other variation in bile duct cell integrity, controlling the amount of absorbed PLE spilling over into the systemic circulation.
- The effect of PLE then depends on incident sunlight, and on the degree of skin pigmentation.

### ACKNOWLEDGEMENTS

This research was funded by DairyNZ and Meat and Wool New Zealand (cattle), and by Ovita Ltd. (sheep). We thank the six dairy farmers and the beef farmer for allowing us access to animals in their herds, and Kevin Knowler, Manager of AgResearch's Woodlands Farm, for care of the sheep. We also thank AgResearch staff in the Waikato and at Woodlands for obtaining blood samples from the animals studied, and Peter Dobbie at AgResearch, Ruakura for the PLE assays.

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