Detection of toxicity in ruminants consuming leucaena (*Leucaena leucocephala*) using a urine colorimetric test

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Introduction

Leucaena *(Leucaena leucocephala)*, a productive leguminous shrub for feeding ruminant livestock, contains the toxic amino acid, mimosine which post-ingestion is converted to 3,4-DHP and 2,3-DHP, isomers of dihydroxy-pyridone. While DHP generally does not exhibit acute toxic symptoms, it has been suggested that it is an appetite suppressant that reduces animal live weight gain (Jones 1994). With no observable symptoms, subclinical toxicity is difficult to detect (Phaikaew *et al*. 2012). In 1982 the DHP-degrading rumen bacterium named *Synergistes jonesii* was introduced into Australia as a potential solution to DHP toxicity as it spreads easily throughout cattle herds grazing leucaena (Jones 1994). However, toxicity events reported since the 2003 drought suggest that the toxicity status of herds, previously understood as being protected, may have changed. This may be the result of loss of effective *S. jonesii* bacteria from the rumen. Widespread subclinical leucaena toxicity has since been confirmed representing a significant economic threat to the beef industry (Dalzell *et al*. 2012).

At present the testing for toxicity requires a sophisticated chemical analysis of urine samples using high performance liquid chromatography (HPLC). Producers, however, require a robust and reliable means to routinely test for toxicity in their herds. A colorimetric urine test protocol is available based on the colour reaction of mimosine and DHP with $FeCl_3$ solution (Jones 1997). When this simpler colorimetric test has been used under a wide range of conditions false negatives have been reported. The aim of this study was to improve the reliability of the $FeCL_3$ urine colour test.

Materials and Methods

Urine samples collected from 5 herds grazing leucaena pastures in central Queensland were preserved (19 mL urine + 1 mL 10 N HCl). An acid titration was used to determine urine alkalinity. Urine hydrolysis and clean-up methods (filtering & chromatography) were optimized to reduce interference of background compounds. Colour reaction matrix conditions were optimized for the detection of mimosine and 2,3-DHP by adjusting $FeCl_3$ concentration (Fig. 1) and acid (HCl) strength (Fig. 2). Final ratios of urine:$FeCl_3$ reagent were studied to optimize sensitivity of the test. HPLC analysis (Dalzell *et al*. 2012) was performed on urine samples to determine toxin concentrations and validate the colour responses of the test kit.

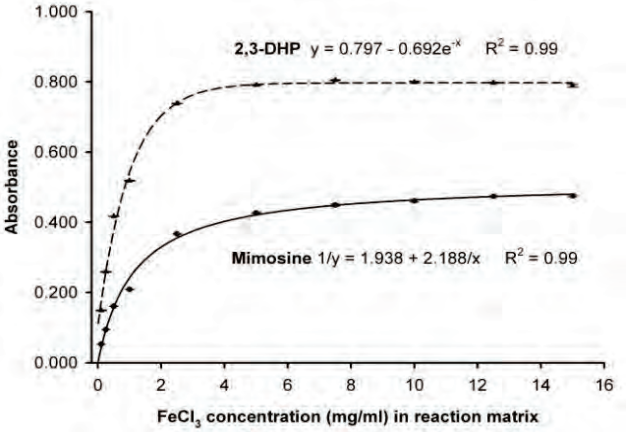

Figure 1. Colour development (absorbance) for 200 µg/mL mimosine (λ=535 nm) and 2,3-DHP (λ=590 nm) at different $FeCl_3$ concentrations in 0.35N HCl

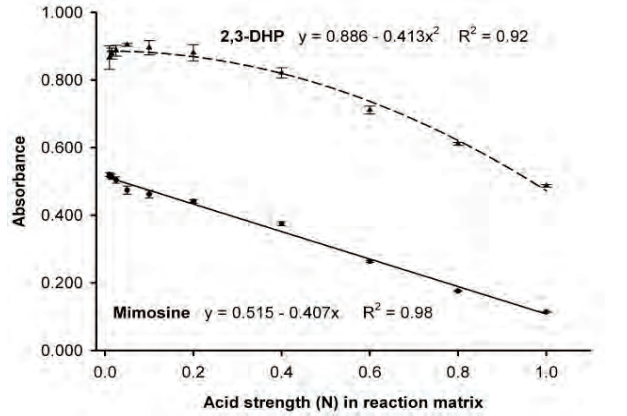

Figure 2. Colour development (absorbance) for 200 µg/mL mimosine (λ=535 nm) and 2,3-DHP (λ=590 nm) at different acid strengths (HCl) with 10 mg/mL $FeCl_3.6H_2O$

Results and Discussion

The urine of cattle grazing leucaena was found to be very alkaline (pH=9). Preserving 9.5 mL urine by adding 0.5 mL 10 N HCl gave a residual acid strength of 0.35 N. Hydrolysis of the preserved urine by heating in boiling

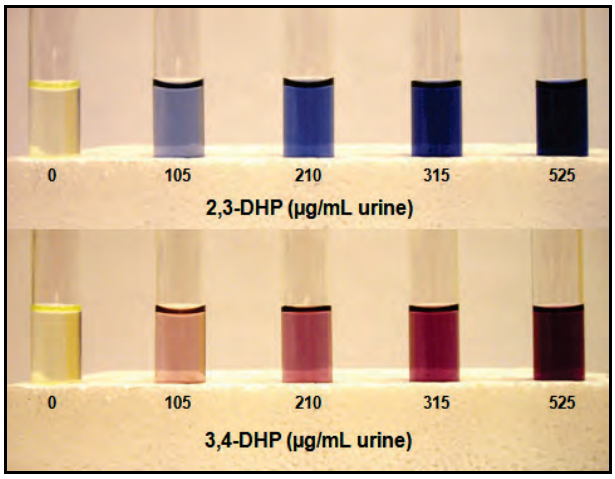

Plate 1. Test response to a range of standards equivalent to µg/mL toxin in urine (1:2 reagent reaction ratio)

water for 1 hour was required to release toxins that are typically conjugated to sugars prior to colorimetric analysis. Hydrolysed samples were then cleaned prior to testing by filtering (0.45 µm) and chromatography (Maxi-Clean 300 mg C-18 columns) to remove background colour. Optimal colour development for both isomers of DHP occurred at $FeCl_3.6H_2O$ concentrations >5 mg/mL and were most consistent at acid strengths of 0.2-0.4 N (Fig. 1 & 2). Colour development for 3,4-DHP was the same as for mimosine (G Kerven unpublished data). A reaction ratio of 1 urine:2 $FeCl_3$ reagent developed good colour without being too sensitive. Samples from commercial cattle herds tested using this procedure developed pink/red colour for mimosine/3,4-DHP and blue for 2,3-DHP (Plate 1). These results were confirmed by HPLC analysis. The colour test proved to be robust with replicated sample results having a coefficient of variation <15%.

When Graham *et al. (*2013) applied this test in the field they found a high level of variability in urinary toxin concentrations among animals within a herd grazing the same leucaena pasture. They recommended that for herds consuming high dietary percentages of leucaena urine samples from at least 10 cattle would be required to reliably assess herd protection status.

The recommended test protocol is: dilute urine samples 19:1 with 10 N HCl; hydrolyse for 1 hour in boiling water; filter (0.45 µm) and pass through a C-18 column; dilute treated urine samples 1:2 with 10 mg/mL $FeCl_3.6H_2O$ in 0.35 N HCl and then compare to standard toxin solutions. If mean herd urinary DHP concentrations >100 µg/mL, the herd may be suffering from leucaena toxicity (Dalzell *et al.* 2012).

Conclusions

This semi-quantitative test kit will enable routine testing of herds for presence of toxins to determine their protection status at relatively low cost. While the urine samples can be collected by farmers, it is likely that the test will be carried out by appropriately trained service providers.

Acknowledgements

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