Determination of the toxic threshold of lolitrem B in cattle eating endophyte-infected perennial ryegrass

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Abstract

Perennial ryegrass (*Lolium perenne*) straw is used as a feed for livestock and horses. Some straw is infected with the endophyte, *Neotyphodium lolii,* which produces lolitrem tremorgens. Ingesting of the toxin produces clinical "ryegrass staggers." A 28 day feeding trail was conducted using 61 Black Angus, pure and crossbred cattle. The cattle were fed one of three levels of lolitrem B in chopped perennial ryegrass straw ranging from 0 ppb up to 3058 ppb. Four Japanese Wagyu crossbred cattle were fed chopped ryegrass straw containing 1400 ppb lolitrem B. The cattle were evaluated and scored twice a day for clinical signs of gait difficulties.

Cattle consuming 1400 ppb lolitrem B did not show any overt clinical signs. After 14 days, 7 out of 15 cattle in the 1954 ppb lolitrem B group showed clinical signs of ataxia, stiffness, and tremors as seen in ryegrass staggers. All of the cattle in the positive control group of up to 3058 ppb lolitrem B with a 4 week mean of 2574 ppb developed clinical signs of staggers. None of the Wagyu cattle at 1400 ppb lolitrem B developed clinical signs. A dose response curve was developed based on the results of this study coupled with a prior study for use in the testing laboratory.

Keywords: lolitrem B, perennial ryegrass, *Lolium perenne,* endophyte fungus, *Neotyphodium lolii,* cattle, threshold levels

Introduction

The endophyte fungus that infects perennial ryegrass (*Lolium perenne*) is *Neotyphodium lolii.* When perennial ryegrass is infected with the endophyte fungus, the beneficial effects are drought resistance, insect resistance, and better production (Clay 1988; Joost 1995; Bush *et al*. 1997). The interaction between endophyte and grass results in varying quantities of lolitrem alkaloids affecting the nervous system of animals who feed on it (Porter 1995; Oliver 2005). In addition, endophyte-infected perennial ryegrass also produces ergovaline, an ergot alkaloid, but in lesser amounts (Hovermale & Craig 2001). The Pacific Northwest of the United States produces 75% of the world's supply of perennial ryegrass. With the cessation of field burning of straw residues, a large amount of ryegrass straw became available for animal feed. In 2005, 660 000 tons were shipped to Japan, Korea, and Taiwan. The Japanese use this straw in a mix of feed for Japanese black cattle because of its fibre content and the straws' ability to produce white fat (due to its lack of carotenes). In addition, this ryegrass straw is used as a fibre source in Japanese dairies.

The lolitrem alkaloids in endophyte-infected perennial ryegrass, particularly lolitrem B, produce a reversible clinical condition called "ryegrass staggers" when the quantity of lolitrem B is above a certain level, termed "threshold level." Clinical signs include an initial stiffness of gait which progresses to incoordination (ataxia), muscle weakness, and tremors. The tremors begin in the large muscles of limbs as animals contract them to stand and walk. They can progress to muscle tremors in the neck and head. If feeding endophyte-infected perennial ryegrass continues, animals soon become unable to rise even though they struggle to do so. On the cessation of the feeding of endophyte-infected perennial ryegrass the animals showed improvement in 2 to 4 days and in most cases return to normal (Smith 2002). Postmortem evaluations of affected animals show no morphological lesions (Hunt *et al*. 1983).

Determination of a threshold level of lolitrem B causing ryegrass staggers became possible when an analytical assay for the molecule and pure standards became available (Gallagher *et al*. 1985; Miles *et al*. 1992; Hovermale & Craig 2001). Some of the first studies determined lolitrem B levels in pastures where clinical cases of ryegrass staggers were seen (Galey *et al*. 1991; di Menna *et al*. 1992; Tor-Agbidye *et al*. 2001).

From these studies and others, a special panel of scientists (1999-2000) met to set the toxic threshold level at 2000 ppb for cattle and sheep. This threshold level has been used to determine which straw would be exported to Asian countries. Subsequently, reports of Japanese black cattle being affected by lolitrem B levels lower than this threshold level came to the attention of the grass straw industry (Saiga & Maejima 1998; Saiga 1998; Miyazaki *et al*. 1999; Miyazaki *et al*. 2001). Feeding trials were set up to deliberatively feed endophyte-infected perennial ryegrass straw with known levels of toxin to cattle. In the pilot study (Fisher *et al*. 2004), 72 Angus/Hereford cross bred cattle were fed perennial ryegrass straw and supplemented with soybean meal during the last trimester of their pregnancy. With perennial ryegrass straw at approximately 2000 ppb (2017 ppb), 54% of the cattle developed ryegrass staggers while cattle ingesting perennial ryegrass straw containing 1550 ppb or lower over a period of 3 months did not. A second study which is reported here was conducted to further define the lolitrem B threshold that causes ryegrass staggers.

Figure 1 Ataxia and proprioceptive deficits seen in a steer with ryegrass staggers.

Table 1 Scoring system.[a]

Score	Clinical signs
0	No clinical signs
1	No resting tremors or incoordination; low-intensity tremor and incoordination with handling; slight stiffness of gait
2	No resting tremors or incoordination; moderate-intensity tremors and incoordination with handling; marked stiffness of gait
3[b]	Spontaneous low-intensity tremors and incoordination at rest; moderate to severe tremors and incoordination with handling; marked stiffness of gait; difficulty in rising
4	Pronounced resting tremors and incoordination; convulsive tremors and severe incoordination with handling; extreme spastic gait (goose stepping)
5	Severe spontaneous tremors and incoordination at rest, usually accompanied by collapse into lateral recumbency and convulsive episodes

[a]Adapted from Galey *et al*. (1991).
[b]Removal from the study occurred at a scale score of 2 or higher.

Table 2 Clinical response to ingestion of perennial ryegrass with varying lolitrem B concentrations in Black Angus and Black Angus cross bred cattle from this study and a companion study by authors (Fisher *et al*. 2004) are detailed. The number of animals affected with ryegrass staggers over the number of animals in each feeding group is listed.

Lolitrem B conc. (ppb)	Blythe *et al.*	Fisher *et al.*
0 (<100 ppb)	0/16	0/24
1400	0/15	
1550		0/24
1954	7/15	
2017		13/24*
2574 - 3058	15/15	

* One death

Materials and Methods

Sixty five cattle were used for this study. Fifty one were Angus and 10 Angus/Hereford cross bred cattle between 11 and 13 months of age (32 heifers and 29 steers). They were placed in pens containing five animals each except for one pen which contained six animals. The additional four were Japanese black cattle/black Angus crossbred cattle (75%:25%) who were penned separate from the others. Straw at three levels of lolitrem B were fed to three groups of 15 animals. The control was a group of 16 animals. Group 1 were fed 1398 ppb lolitrem B straw; Group 2 were fed 1954 ppb lolitrem B straw; Group 3 started the 28 day trial with 1845 ppb lolitrem B for week 1, 2435 ppb lolitrem B for week 2, 2957 ppb lolitrem B for week 3, and finally, 3058 ppb lolitrem B for week 4. Note weeks 3 and 4 were basically the same level. The mean ppb lolitrem B for Group 3 for the 4 weeks was 2574 ppb. Group 4 was the control group with 0 ppb lolitrem B. The four Japanese black cattle were fed the same straw as Group 1 with 1398 ppb lolitrem B. The grain mix consisted of crushed corn and soy grits and was given to each animal at the rate of 0.7 kg per head per day before feeding the straw.

Cattle were fed twice a day at 8 am and 4 pm. The alkaloid values in the straw from a field can vary by up to 30%. To insure uniform feed, the compressed bales of straw (approximately 350 kg each) were mixed in a dairy mixer for 30 minutes. Fibre length of the straw after mixing was approximately 10 cm. Approximately 4.5 kg of straw was fed per animal per feeding to assure that no pen was completely devoid of straw by the next feeding. Any straw not consumed (orts) was collected, weighed, and sampled for lolitrem B. The four Japanese black cattle were fed in the same manner except they had an additional 0.45 kg of crushed corn, oats, and barley mixed grain per animal per feeding. All animals had unrestricted access to fresh water and a trace mineralised salt block. For 3 weeks before the start of the feeding trial, all cattle were fed endophyte negative perennial ryegrass straw *ad libitium* with 0.7 kg of crushed corn and soy grit.

Straw was assayed for lolitrem B at the time of feeding and weekly throughout the 28 day trial. The orts were collected daily and served as a monitor of the daily feed intake of lolitrem B. Feed was weighed prior to feeding and left over straw was also weighed to get a total feed intake per pen per day.

Clinical examination of each animal by two of the investigators was done twice a day. Investigators were blind to which group had the high, medium, low, and no lolitrem B. The Japanese black cattle were known to be getting approximately 1400 ppb lolitrem B in their straw, but they were examined in the same manner. Each animal was examined as they moved around the pen and individually walked up and down a 46 m alley at 7 am and 3 pm each day. A scoring system of clinical signs adapted from Galey *et al*. (1991) was used (Table 1). When an animal scored 2 or higher, it was removed from the study and placed on an endophyte-free pasture. Respiratory rates at rest were monitored twice a week, just before walking. At the end of the study, a fat biopsy was taken under a local anesthetic block from the tail head of three animals in Group 4 (controls) and two animals in the high lolitrem B Group 3.

Straw was ground to 0.5 mm and partitioned into a chloroform methanol mixture before analysis for lolitrem B. After shaking for 18 hours, the mixture is evaporated to dryness, reconstituted into a chloroform/acetonitrile mixture, and quantitated by an HPLC fluorescence assay (Hovermale & Craig 2001)

Analysis for lolitrem B in fat used the method of Miyazaki *et al*. (2004). In short, it was a solvent extraction with chloroform with HPLC fluorescence detection. Quantitation was made by comparison to lolitrem B crystalline standards.

Data was analysed by randomised blocks using GLM procedures from SAS.

Table 3 Lolitrem B concentrations in cow tissues (Miyazaki *et al*. 2004).

Cow		Feeding	---------- Lolitrem B (ppb in wet tissue) ----------					
Group	No.	duration	Muscle	Liver	Kidney	Heart	Cerebrum	Fat
1	1	15 d	ND*	ND	ND	ND	ND	210
2	1	5 wk	ND	ND	ND	ND	NT†	130
	2	13 wk	ND	ND	ND	ND	NT	123
	3	16 wk	ND	ND	ND	ND	NT	144

* ND = not detected.
† NT = not tested.

Results

Table 2 shows the clinical results of the study. All animals scored 0 in the first 12 days of the study. Initially, the lolitrem B content of the mixed straw in the positive control Group 3 was found to be less than the amount set in the protocol (2500 ppb) at the end of week 1, so the feed was replaced with feed that averaged 2435 on week 2. Clinical signs were seen in the positive control Group 3 in four animals at a grade of 2 at the end of 2 weeks; they were removed from the study. At the end of week 3, six additional animals scored between 2 and 3 and were removed from the study. By the end of the study, all the remaining Group 3 (high group) animals scored 3. Since the clinical signs were first evident only at the end of the second week in the high level lolitrem B positive control group and in anticipation of the fat biopsies, lolilltrem B in Group 3 straw was increased to approximately 3000 ppb (2957 ppb for week 3 and 3058 ppb for week 4). The mean of lolitrem B over the 4 week period was 2574 ppb.

Seven of the15 cattle in Group 2 with 1954 ppb of lolitrem B were showing clinical signs of stiffness of gait by the end of week 2 with increasing severity of signs up to grade 3 in week 3. All animals who reached a clinical score of grade 2 to 3, were removed from the feeding trial (Fig. 1). Cattle in Group 1 consuming approximately 1400 ppb lolitrem B did not show any clinical signs except for slight stiffness of gait in week 4 but no ataxia, muscle tremors, or proprioceptive deficits. All cattle in the negative control group remained clinically normal. Likewise, the four Japanese black cattle ingesting straw with 1400 ppb lolitrem B for the 4 week period did not develop any clinical signs.

The mean lolitrem B from triplicate assays on fat in one animal on the high lolitrem B feed (high of 3058 ppb and mean of 2574 ppb over 4 weeks) was 71.0 ppb with a SD of 15.3 ppb. In the second animal on the same feed, the fat level of lolitrem B was 61.0 ppb with a SD of 15.9 ppb. The fat from three control animals with no lolitrem B were negative for lolitrem B with the level of detection being 11.1 ppb and the level of quantitation being 37.0 ppb.

Discussion

Cattle consuming 1400 ppb of lolitrem B perennial ryegrass straw for 28 days did not develop overt clinical signs of ryegrass staggers. The stiffness in gait was subjective and vacillated from being present and not being evident during successive evaluations. Fisher *et al*. (2004) did not detect clinical signs of ryegrass staggers at 1550 ppb of lolitrem B or at lower doses over 3 months in pregnant Angus/Hereford cross bred cattle. Di Menna *et al*. (1992) found that 2000 to 2500 ppb lolitrem B in perennial ryegrass pastures produced ryegrass staggers in lambs, but pasture grasses with 1600 ppb lolitrem B did not. Galey *et al*. (1991) found that a perennial ryegrass field with lolitrem B at 3000 ppb in the grass base produced clinical ryegrass staggers in sheep. A ryegrass field being grazed in Oregon produced clinical cases of ryegrass staggers in sheep when the lolitrem B level of the grass was 2135 ppb but clinical signs were not seen when sheep grazed a pasture with 1465 ppb (Tor-Agbidye *et al*. 2001).

In contrast, Miyazaki *et al*. (1999) reported on 29 cases of ryegrass staggers in cattle and horses in Japan with lolitrem B ranging from 970 ppb to 3700 ppb. In a subsequent publication (2004), they found ryegrass staggers occurring in three Japanese black cattle after 12 to 15 days of ingesting perennial ryegrass straw containing 1200 ppb lolitrem B as the only feed source. In the same study, three Japanese black cattle fed 50% of the 1200 ppb lolitrem B perennial ryegrass straw, did not develop clinical signs. The lolitrem B assays were all done in Japan with a different analytical technique and compared to assay values used in the United States which may or may not account for the difference in threshold levels. Another factor could be an increased susceptibility of purebred Japanese black cattle. The four Japanese black crossbred cattle used in this study were 75% Japanese black cattle and 25% Black Angus cross bred which may have contributed to the difference.

Length of time consuming lolitrem B feed may also play a role. While lolitrem B is not thought to be a cumulative toxin and clinical signs of disease disappear after several days off the feed, continued feeding of levels below the established threshold value of 1800 to 2000 ppb lolitrem B may produce disease (Miyazaki *et al*. 2004; Miyazaki personal communication). However, Fisher *et al*. (2004) found no signs of ryegrass staggers in pregnant cows fed 1550 ppb for 3 months. In addition, with establishment of threshold levels for imported straw from the United States, occurrence of ryegrass staggers is now rare in Japan (Miyazaki *et al*. 2004).

The fat levels of lolitrem B in this study were 51 ppb and 61 ppb after 28 days of feeding the high lolitrem B straw (mean of 2574 ppb lolitrem B). This is in contrast to the higher fat levels found in the Japanese study with lower levels of lolitrem B in the straw but fed over a longer period of time (Table 3). While this latter study did not find lolitrem B in any other tissues, its presence in the fat at subclinical doses of lolitrem B, may present a public health concern or may be a non-significant finding relative to human health. Further studies need to be conducted to answer this question.

With increasing levels of endophyte in Pacific Northwest perennial ryegrass, the determination of “threshold levels” has major economic impact on the grass seed industry as well as the Japanese cattle markets that need this source of fibre.

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