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Fluoroacetate toxicity protection in cattle

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Abstract

Twenty Friesian cross cattle were divided into two groups of ten, by pairwise matching of weights. Test animals were orally inoculated with 50 ml each of *Butyrivibrio fibrisolvens* strains OB156, OB291, OR85, 0/10, S2/10, 149/33, and 10/1, all carrying recombinant plasmid pBHf. The fluoroacetate dehalogenase gene carried on this plasmid enables the bacteria to detoxify fluoroacetate. Rumen samples were collected on days 7, 12, and 15 after inoculation, to confirm by Polymerase Chain Reaction the presence of bacteria carrying the dehalogenase gene.

All animals were challenged with sodium monofluoroacetate (3 equal doses at 0, 3, and 6 h, totalling 0.33 mg/kg body weight: calculated as $1.1 \times LD_{50}$) on day 19 and monitored continuously for 24 h. Water was provided *ad libitum* and feed was provided at approximately 14 h.

Genetically modified (GM) bacteria were present at $>10^5$ cells/ml of rumen contents (4 animals), $>10^6$/ml (5) and $>10^7$ cells/ml (1). Strains OR85, 149/33, OB291 and S2/10 successfully colonised the cattle rumen.

Five control animals displayed acute toxicity symptoms between 11 h – 15:30 h and were euthanased. The remaining five displayed reduced activity and periods of reduced rumination, but were visibly recovering by 24 h.

No inoculated animals showed acute signs of toxicity. All showed reduced activity, but continued to ruminate when not sleeping. All appeared normal at 24 h.

Symptoms of fatal toxicity in 50% of the control animals matched the outcome predicted for $1.1 \times LD_{50}$. The absence of acute symptoms in all inoculated cattle represented a significant reduction in toxicity ($P = 0.03$).

Executive Summary

Background

The fluoroacetate detoxification project was initiated by a group of animal producers, who formed the consortium Applied Biotechnology Ltd (Qld) for the purpose of finding a solution to the poisoning of livestock by the Georgina gidgee (*Acacia georginae*). Throughout the Georgina river basin of Western Queensland and the Northern Territory, around 200 cattle per year are lost to poisoning on each property (personal communication with property owners). In northern Queensland the heartleaf tree (*Gastrolobium grandiflorum*) is more acutely toxic and cattle losses occur through accidental contact of animals with the toxic plant. Similar heartleaf poisoning of livestock occurs across the north of the Northern Territory, north of Western Australia, and the southeast of WA. In the southeast of WA, toxicity of the fluoroacetate producing plants (*Gastrolobium spp.*) is extremely high. Through the commitment of producers to contribute their own funds to this research, MLA (previously AMLRDC and MRC) agreed to fund the major portion of the research.

Previous Research

Four strains of rumen bacteria (*Butyrivibrio fibrisolvens*) had been genetically modified by inserting a gene from a soil bacterium (*Moraxella species*)[1]. The gene enabled the rumen bacteria to produce an active enzyme (fluoroacetate dehalogenase) that could break down the toxic compound[2]. The efficacy of the four strains at reducing the effects of fluoroacetate on sheep was demonstrated in contained trials[3] at the University of New England in 1996 – 1997. Subsequent work by the Rumen Biotech group at Murdoch University extended the number of *B. fibrisolvens* strains capable of detoxifying the poison to seven, with an accumulated detoxifying capability approximately twice that of the original four strains.

Tracking primers were developed for all seven strains. In additional work, it was shown that none of these seven strains could colonise the digestive tracts of rabbits or cats, relieving the concern that non-ruminants might be protected from the control agent Compound 1080. Two modified *Bacteroides* strains that produced the detoxifying enzyme and did colonise the digestive tract of rabbits and cats were shown to provide no protection to rabbits or cats. This was predictable from the different digestive physiology of non-ruminants, in which there is no pregastric fermentation. With non-ruminants the animal absorbs the toxin before it reaches the parts of the digestive tract that contain significant numbers of bacteria.

Field trials with non-GM rumen bacteria proved that they are capable of transfer between animals over distances up to 30 metres. The GM regulatory body Genetic Manipulation Advisory Committee (GMAC) therefore recommended that the planned field trials of toxicity protection in ruminants should be transferred to a biosafety containment (level PC2 classified) indoor facility. The CSIRO facility at Werribee was selected as the most suitable venue.

Summary of Results

1. Cattle inoculated with a mixture of bacteria were colonised by strains OR85, 149/33, OB291 and S2/10 at $>10^6$ cells/ml rumen fluid. The recombinant strains 0/10, 10/1 and OB156 were rarely detected within rumen contents.
2. All cattle challenged with 0.33 mg/kg body weight of sodium monofluoroacetate, administered in three identical sub-doses at times 0, 3, and 6 h, showed no toxicity symptoms before 6 h, and only mild symptoms before 9 h.

3. Five control animals showed acute symptoms of toxicity, were judged to be unrecoverably intoxicated (recumbent and showing violent neurological signs), and were euthanased at approximately 11 h, 13 h, 15, 15:15 h and 15:30 h.
4. The remaining five control animals showed reduced movement and reduction in rumination during the period 6 h to 20 h.
5. All of the ten test animals showed some reduction in activity, during the period from 9 h to 15 h, but continued to ruminate at a normal level.
6. None of the test animals showed acute symptoms of toxicity.

Benefits to Industry

At the conclusion of this trial, it is clear that modified rumen bacteria are capable of reducing toxic effects of fluoroacetate in cattle. Field use of the technology will now depend on approval for release by Office of the Gene Technology Regulator (OGTR*).

If released for use, this technology cold benefit the animal producers in the Georgina river basin, through the Queensland heartleaf regions and in many parts of northern Australia and Western Australia. Economic losses from fluoroacetate poisoning are significant, with 200 cattle per year representing at least $120,000 loss p.a. on affected properties (personal communication with property owners).

The bacteria do not provide total immunity to the toxin, but greatly reduce the risk of mortality in poisoned animals. Animals that consume novel poisonous plants and survive are known to develop an aversion to those plants, avoiding them in the future[4,5]. Where familiar browse plants contain persistent low levels of toxin, the likelihood of animals reaching a lethal dose would be significantly reduced, or possibly eliminated.

***Footnote:** The regulatory body GMAC was replaced by the newly created government department OGTR on June 21, 2001

Contents

1 Introduction

Previous research funded by MLA (MRC & AMLRDC) has developed tools for genetic manipulation of the rumen bacterium *B. fibrisolvens*[6]. That work culminated in the development of seven strains that produce the fluoroacetate detoxifying enzyme[2, 3] (Final Report Projects TR.044 & TR.044B). The efficacy of four strains in protecting sheep from the toxin has been demonstrated[3], but demonstration of toxin protection in significant numbers of cattle has been delayed somewhat by safety concerns and the need to meet OGTR licencing conditions and guidelines in conducting such a trial.

The present work was conducted under strict PC2 containment, testing the ability of the GM bacteria to combat a potentially lethal dose of fluoroacetate in cattle.

2 Materials and Methods

The animal experiments reported in this document were approved by the Australian Animal Health Laboratory 'Animal Ethics and Experimentation Committee' (Approval No. AEC 938, March 2003).

Bacterial strains. Seven strains of *B. fibrisolvens* (10/1, OB156, OB291, OR85, 0/10, S2/10, and 149/33) transformed with the recombinant shuttle plasmid pBHf[2] were grown in rumen fluid medium to stationary phase. The development and testing of these strains has been described in the Final Report of MLA Project TR.044B. Prior to shipping to the Australian Animal Health Laboratories (Vic) all strains were tested for production of fluoroacetate dehalogenase activity at the Rumen Biotech laboratories at Murdoch University.

Cattle maintenance and oral inoculation. Twenty Friesian crossbred steers were housed in a PC2 animal room and acclimatised for 2-3 days, with a diet of oaten chaff and lucerne chaff (3:1) and water provided *ad lib.* During the toxin challenge period animals were fitted with Polar Accurex Plus (TM) heart-rate monitors, which were attached to a girth-strap. The ten test animals were inoculated orally with an equal mixture of the seven bacterial cultures (50 ml of each strain). Rumen samples were removed by stomach tube from both control and test groups and were tested for the presence of recombinant bacteria at days 7, 12 and 15 days, post-inoculation. Following fluoroacetate poisoning, rumen fluid from euthanased animals was collected directly from the dissected rumen (day 20).

Bacterial detection. The numbers of recombinant bacteria in rumen samples were measured by Polymerase Chain Reaction (PCR) amplification of a 300 bp region of the dehalogenase gene from a series of dilutions. Elimination of the PCR product by serial dilution provided a minimum number of target organisms present. Forty microliter PCR reactions were set up with: 2.5 mM $MgCl_2$, 166 μM dNTPs, 1.3 μM each of primers CC5 and CC6.2i and 1 μl of target; and amplification by 2 cycles of 95°C-5 min, 55 °C-1 min, 30 cycles of 72 °C-1 min; 95 °C-1 min, 55 °C-1 min, 72 °C-1 min; and an additional cycle of 95 °C -1 min, 55°C -1 min and 72 - 8 min. Ten microlitres of the PCR product was run on a 2% agarose gel (0.5 x TBE) with ethidium bromide, and analysed on a Bio-Rad Gel-Doc 2000 system.

Toxin challenge. Toxin challenge was commenced at day 20. Fluoroacetate doses were calculated for each animal on the basis of body weight, to provide 0.33 mg/kg body weight. Solutions were adjusted to allow 0.11 mg/kg to be administered per dose and doses were fed to animals at times 0, 3, and 6 h of the challenge period. Fluoroacetate dissolved in water was applied to bread flavoured with molasses, which was accepted voluntarily throughout the dosing period.

Assessment of toxic effects. Animals were monitored continuously from 0 h – 24 h from the first fluoroacetate dose. Records were kept of behaviour: standing/lying, feeding, drinking, ruminating, general demeanour, startle reflex, and attention to movement around them. Acute

signs of toxicity were responded to by immediate delivery of the sedative xylazine and injection with the euthanasia drug sodium phenobarbitone.

3 Results

All animals were accustomed to the presence of research staff prior to the toxicity trial. Rumen samples were negative by PCR for presence of the recombinant dehalogenase gene in the control animals, and in test animals prior to inoculation. Total numbers of recombinant fluoroacetate degrading bacteria present in each animal following inoculation are shown in Table 1.

Table 1. No. of fluoroacetate degrading bacteria present in each animal at 3 collection times.

	Total number of fluoroacetate degrading strains (cells/ml)		
Test animal	Day 7	Day 15	Day 20
1	10^5	10^5	10^7
2	10^5	10^5	10^5
3	10^4	10^5	10^6
4	10^5	10^5	10^6
5	10^5	10^5	10^5
6	10^6	10^5	10^6
7	10^5	10^5	10^6
8	10^4	10^5	10^6
9	10^4	10^5	10^5
10	10^5	10^5	10^5

PCR analysis of the crude rumen sample taken at the end of the trial indicated that all cattle inoculated with the mixture of bacteria were colonised by strain 149/33 and S2/10; eight of the animals contained strains OR85 and OB291; strains 0/10 and OB156 were present at 10^4/ml for only one animal; strain 10/1 was not detected within rumen contents (Table 2). The levels of colonisation based on PCR analysis of the crude rumen contents ranged from 10^4 to > 10^6 for strains OR85, 149/33, 291 and S2/10.

Table 2. Fluoroacetate degrading bacterial strains present in each animal at termination of trial (day 20 post inoculation).

	Strains of fluoroacetate degrading bacteria						
Test Animal	0/10	10/1	OR85	149/33	OB156	OB291	S2/10
1	10^4	-	10^6	10^6	10^4	10^6	10^4
2	-	-	10^4	10^4	-	10^4	10^4
3	-	-	10^6	10^4	-	10^4	10^4
4	-	-	10^6	10^4	-	10^6	10^6
5	-	-	10^6	10^6	-	10^6	10^6
6	-	-	10^6	10^4	-	10^6	10^4
7	-	-	10^4	10^4	-	10^4	10^4
8	-	-	10^4	10^4	-	10^4	10^4
9	-	-	-	10^4	-	-	10^4
10	-	-	-	10^4	-	-	10^4

(−) strain absent.

Post-mortem rumen fluid samples were also serially diluted (ten fold) up to 10^{-6} into anaerobic media supplemented with 200 μg/ml erythromycin (fluoroacetate degrading strains are resistant), enriched and the resultant cultures were analysed for the predominant fluoroacetate degrading

strains by PCR. This analysis indicated that all cattle had been colonised with strains OR 85 and OB 291 at > 10^6 /ml. Several of the animals also contained strains 149/33 and S2/10 at this level as well (Table 3). All other strains were absent at this dilution.

Table 3. Presence of fluoroacetate degrading strains at 10^{-6} dilution of enriched post-mortem rumen

	Fluoroacetate degrading strains			
Test animal	OR85	149/33	OB291	S2/10
1	+	+	+	+
2	+	-	+	+
3	+	-	+	-
4	+	-	+	+
5	+	-	+	+
6	+	-	+	-
7	+	+	+	+
8	+	+	+	+
9	+	-	+	+
10	+	-	+	-

(+) strain present; (−) strain absent.

All cattle challenged with sodium monofluoroacetate, in three identical doses at times 0, 3, and 6 h, showed no toxicity symptoms before 6 h. Five control animals showed reduced movement and reduction in rumination during the period 6 h to 20 h.

The remaining five control animals showed sudden severe symptoms of toxicity (un-coordinated, recumbent, unable to stand, paddling action) were judged to be unrecoverably intoxicated, and were immediately euthenased at approximately 11 h, 13 h, 15, 15:15 h and 15:30 h respectively. All the other control animals were very lethargic and depressed from 6h to 20h.

All ten test animals showed some reduction in activity, during the period from 9 h to 15 h, but continued to ruminate at a normal level. The test animals appeared to be more alert and not as depressed as the control animals and were better co-ordinated in their movements. None of these animals became recumbent with an inability to stand.

None of the test animals showed acute symptoms of toxicity. The protective effect of the GM bacteria was calculated to be significant using the Fisher's exact test (P = 0.03).

3.1 Discussion

Many attempts to re-colonise the rumen with laboratory grown bacteria have proven unsuccessful (see review)[7]. However in previous work, strains of rumen bacteria that were stored with minimum culture cycling were shown capable of recolonising the rumen on a long-term basis[8]. The strains used in this study included two strains from Canadian white-tail deer (OB156, OB291), two strains from Australian sheep in NSW (10/1) and WA (S2/10). The remaining three strains were isolated from cattle in Canada (OR85) or WA (0/10, 149/33). It was clear from the population numbers achieved here that successful colonisation of ruminant digestive systems by laboratory grown bacteria was not impaired by the presence of a recombinant plasmid within the bacteria.

The effects of fluoroacetate on heart-rate was measured successfully for 5 of the control animals, but the monitors failed to yield results from the test animals, partly through their more prolonged interest in chewing the fittings and the equipment. Comparisons between groups were therefore not possible.

The fluoroacetate dose administered to cattle in this experiment was calculated to be 10% above the published LD_{50} of 0.3 mg/kg body weight. It was predicted that approximately half of the

animals would show fatal intoxication. In the control group this prediction was met precisely. In the inoculated test group, the complete absence of fatal intoxication was consistent with significant reduction of the fluoroacetate passing from the rumen to the absorptive regions of the gastrointestinal tract. It was concluded that the recombinant bacteria defluorinated a significant proportion of the fluoroacetate present within the rumen fluid.

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4 References

1. **Kawasaki H, Yahara H, Tonomura K.** (1984) Cloning and expression in *Escherichia coli* of the haloacetate dehalogenase genes from *Moraxella* plasmid pUO1. Agric.Biol.Chem. 48: 2627-2632.
2. **Gregg, K., Cooper, C.L., Schafer, D.J., Sharpe, H., Beard, C.E., Allen, G., Xu, J.** (1994) Detoxification of the Plant Toxin Fluoroacetate by a Genetically Modified Rumen Bacterium. Bio/Technology 12:1361-1365.
3. **Gregg, K. Hamdorf, B.J. Henderson, K.P., Kopecny, J., Wong, C.** (1998) Genetically modified rumen bacteria protect sheep from fluoroacetate poisoning. Appl. Environ. Microbiol. 64:3496-3498.
4. **Provenza FD.** (1996). Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. J. Anim. Sci. 74:2010-20.
5. **Ralphs MH, Provenza FD.** (1999) Conditioned food aversions: principles and practices, with special reference to social facilitation. Proc. Nutr. Soc. 58:813-820.
6. **Beard, C.E., Hefford, M.A., Forster, R.J., Sontakke, S.,Teather, R.M., Gregg, K.** (1995) Stable and efficient transformation system for *Butyrivibrio fibrisolvens* OB156. Current Microbiol. 30:105-109.
7. **McSweeney, C.S., Dalrymple, B.P., Gobius, K.S., Kennedy, P.M., Krause, D.O., Mackie R.I., and Xue, G.P.** (1999) The application of rumen biotechnology to improve the nutritive value of fibrous feedstuffs: Pre and post ingestion. *Livestock Production Science* 59: 265-283.
8. **Gregg, K., Allen, G., Beard, C.** (1996) Genetic manipulation of rumen bacteria: from potential to reality. Aust. J. Agric. Res. 47:247-256.