CASE REPORT

Aspergillus clavatus tremorgenic neurotoxicosis in cattle fed sprouted grains

RA McKENZIE[a], MA KELLY[a], RG SHIVAS[b], JA GIBSON[c], PJ COOK[d], K WIDDERICK[e] and AF GUILFOYLE[f]

Beef and dairy cattle from four different herds in southern and central Queensland fed hydroponically-produced sprouted barley or wheat grain heavily infested with *Aspergillus clavatus* developed posterior ataxia with knuckling of fetlocks, muscular tremors and recumbency, but maintained appetite. A few animals variously had reduced milk production, hyperaesthesia, drooling of saliva, hypermetria of hind limbs or muscle spasms. Degeneration of large neurones was seen in the brain stem and spinal cord grey matter. The syndrome was consistent with *A clavatus* tremorgenic mycotoxicosis of ruminants. The cases are the earliest known to be associated with this fungus in Australia. They highlight a potential hazard of hydroponic fodder production systems, which appear to favour *A clavatus* growth on sprouted grain, exacerbated in some cases by equipment malfunctions that increase operating temperatures.

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Aspergillus clavatus is a cosmopolitan soil fungus commonly isolated from many cereal grains and their malts (germinated seeds) including barley, maize and sorghum, as well as other stored feedstuffs, including legume seeds.[1,2] It occurs frequently in pigeon droppings,[1] and is considered a major cause of the human extrinsic allergic alveolitis known as 'malt worker's lung'.[1,2] Its spores are carcinogenic in mouse lung.[3]

A tremorgenic mycotoxicosis from eating sprouted grain infested with *A clavatus* has been described in ruminants in Europe, South Africa, China, Israel and Brazil.[4-11] Sprouted grains of barley, wheat, sorghum, and maize have been involved. We report here three cases from south-eastern Queensland and one from central Queensland in cattle fed sprouted barley or wheat to record the first known occurrence of this syndrome in cattle in Australia. A preliminary record of one subsequent probable case in feedlot sheep in Australia has been published.[12]

Laboratory methods

Clinical chemistry profiles of serum were obtained using an automated analytical system (Olympus Reply®). Calcium, magnesium, total protein, total bilirubin, creatinine, urea, γ-glutamyl transferase, creatine kinase and aspartate aminotransferase were assayed using commercial kits (Thermo Trace, South Oakleigh, Victoria) and albumin and glutamate dehydrogenase with other kits (Randox Laboratories Ltd, Antrim, UK). Haematological profiles were obtained by microhaematocrit and automated electronic examinations for haemoglobin, packed cell volume, erythrocyte and leucocyte counts and erythrocyte indices. Reference ranges used for results interpretation were those established at the Yeerongpilly Veterinary Laboratory. Tissue samples were fixed in 10% formalin, processed by routine methods and haematoxylin-eosin sections were examined by light microscopy. Suitable brainstem tissue was examined in line with the Australian Diagnostic Standard for Transmissible Spongiform Encephalopathy (TSE) Exclusion.[13] *A clavatus* was identified directly from sprouted grain samples by recognising the characteristically clavate (club-shaped) conidial heads in wet mounts of the fungus by light microscopy.[14] Serum was tested for the presence of *Clostridium botulinum* toxin types C and D by ELISA.[15]

Case histories and laboratory findings

Case 1 (Contributing authors PJC, RAM, MAK)

A herd of 45 Brahman-cross steers, 23 months old, was examined near Kilcoy on 23 September 2002. They were grazing a kikuyu (*Pennisetum clandestimum*) and white clover (*Trifolium repens*) pasture with a very small amount of ryegrass (*Lolium* sp) and were also fed grass hay and sprouted barley grain (*Hordeum vulgare*) 4 days after germination in a 'fodder factory' - an automated hydroponic production system housed in a climate-controlled shed. Two weeks before onset of signs on 20 September 2002, the computer controlling the shed environment had malfunctioned, allowing the temperature in the facility to increase to 50°C and the humidity to increase. A heavy growth of *A clavatus* was identified among the grains in a sample of sprouted barley (Figure 1) collected on 23 September from the batches fed to affected cattle. A specimen was deposited in the Department of Primary Industries Plant Pathology culture collection as accession BRIP39262a. One steer was dead and eight others were ataxic, with knuckling of hind fetlocks, or in sternal recumbency, but were still eating and drinking.

Jugular blood samples were taken from two recumbent steers for haematological and clinical chemistry profiling. Haematological findings were polycythaemia, interpreted as due to dehydration (erythrocyte counts 10.1 and 10.2 x 10^{12}/L; reference range 5.8 to 8.9), hypochromic and microcytic erythrocytes (mean cell volumes 42 and 39 fL; reference range 44 to 55; mean cell haemoglobins 13 and 11.8 pg; reference range 14 to 20) and slight to moderate neutrophilias (neutrophil counts 11.9 and 5.7 x 10^{9}/L ; reference range 1.2 to 4.0). Clinical chemistry findings were increased activities of creatine kinase (12980 and 4112 IU/L; reference range 10 to 200) and aspartate aminotransferase

[a]Queensland Department of Primary Industries, Yeerongpilly Veterinary Laboratory, Animal Research Institute, Locked Mail Bag No.4, Moorooka, Queensland 4105. E-mail: ross.mckenzie@dpi.qld.gov.au
[b]Queensland Department of Primary Industries, Agency for Food & Fibre Sciences, 80 Meiers Road, Indooroopilly, Queensland 4068
[c]Queensland Department of Primary Industries, Toowoomba Veterinary Laboratory, PO Box 102, Toowoomba, Queensland 4350
[d]5047 D'Aguilar Highway, Kilcoy, Queensland 4515
[e]Stanthorpe Veterinary Care Services, 27 Creek Street, Stanthorpe, Queensland 4380
[f]Laglan Road, Clermont, Queensland 4721

Figure 1. ***Aspergillus clavatus*** **infestation of sprouted barley grain fed to affected cattle (Case 1).**

(699 and 987 IU/L; reference range 30 to 170) with normal values for protein, albumin, globulin, calcium, magnesium, total bilirubin, γ- glutamyltransferase, glutamate dehydrogenase, urea and creatinine. No botulinum toxin was detected in either serum.

One affected steer killed for necropsy had slight pulmonary emphysema only. Liver, kidney, heart and lumbar spinal cord were fixed in formalin and haematoxylin-eosin sections examined. Only the spinal cord had histological lesions, namely necrosis or chromatolysis of individual neurones in the ventral grey matter, but no abnormality was seen in white matter.

The mouldy barley sprouts were withdrawn immediately from the steers' diet and the herd was reinspected on 18 October 2002, at which time one steer was still affected with knuckling of the hind fetlocks, but the others had recovered.

Case 2 (Contributing authors KW, JAG, MAK)
One cow (cow 1) in a herd of 16 Hereford-cross cows, 6 to 8 years old, near Stanthorpe was noted with an unusual gait on 13 January 2003 and suspected by the owner of having mastitis. This cow had a normal mammary gland and milk when examined, but had muscle tremors and mild posterior ataxia. It was treated prophylactically for mastitis and placed in a yard with another cow (cow 2) being treated for mastitis. They were fed sprouted barley grain and lucerne (*Medicago sativa*) hay. The whole herd had been fed sprouted barley grain produced hydroponically in a 'fodder factory' system for a number of months before this incident. Management of periodic fungal infestations of the sprouting grain had been attempted by applying solutions of household bleach (10% sodium hypochlorite). Despite this, the owner reported that it was common for the sprouts to be visibly mouldy. The production facility had last been cleaned and treated in early December 2002.

The owner reported cow 2 to have muscle tremors by the evening of 16 January. It developed posterior ataxia on 17 January, became recumbent and was killed next day. No necropsy was performed.

At the time of the second clinical examination on 18 January, cow 1 had muscle tremors, ataxia exacerbated by exercise and became recumbent when handled, regaining its feet after being allowed to rest. Venous blood had normal haematological values, but had increased activities of aspartate aminotransferase (218 IU/L), glutamate dehydrogenase (66 IU/L; reference range 0 to 40) and γ-glutamyl transferase (57 IU/L; reference range 10 to 35) indicative of mild hepatobiliary damage. Cow 1 progressed to lateral recumbency on 20 January with muscle tremors and spasms of limbs, but was unable to rise. The owner killed her by gunshot to the head and she was necropsied immediately, but no lesions were detected. Specimens of the mid and hind brain with no gross gunshot damage, cervical spinal cord, heart, liver, spleen, lung and lymph node were fixed in formalin. There was mild chromatolysis of neurones in the dorsal vagal nuclei. No lesion suggestive of TSE was detected. No lesion was detected in other tissues.

A sample of the sprouted barley grain fed to the affected cows was collected and a heavy growth of *A clavatus* was detected in it. Subsequently, two other cows were seen with muscle tremors, but recovered, and one other cow died unexamined.

Case 3 (Contributing authors AFG, RAM, MAK)
A herd of Friesian, Friesian-Brown Swiss, Guernsey and Guernsey-Brown Swiss dairy cattle in drought conditions near Clermont were fed sprouted wheat (*Triticum aestivum*) grain ('wheat grass') for about 3 months from mid October 2002 without ill effect. The wheat was grown on hammermilled newspaper in a home-built hydroponic production facility without mechanical ventilation. The owners first noticed the growth of a blue mould among the sprouting grains on about 17 January 2003, but continued to feed the material.

Two weeks later, on 26 January, one lactating adult Friesian cow became ill. She trailed one hind leg and then the other in posterior ataxia, had muscle tremors, drooled saliva, and was hypersensitive to touch over the hindquarters. Her behavior appeared abnormal with apparent fear reactions to unfamiliar objects in her path, shadows and other cows. She was isolated from the herd and then had episodes of muscle tremors and spasms in gradually increasing frequency, but maintained a normal appetite. Sprouted grain was withdrawn from her diet on about 29 January, but her condition deteriorated, muscle spasms became more frequent, the ataxia worsened and she had difficulty rising. Examination of the sprouted grain revealed a heavy growth of *A clavatus*. On 31 January the cow became recumbent, was unable to rise, and died in opisthotonos the next day. She was necropsied, but no lesions were seen. Whole brain and samples of lumbar spinal cord, liver, kidney and lung were fixed in formalin. Karyolysis, central chromatolysis, cytoplasmic vacuolation and necrosis were seen in large neurones of midbrain (Figure 2) and medulla oblongata nuclei and in neurones of the lumbar spinal cord grey matter. No lesion suggestive of TSE was detected. No abnormality was seen in other tissues.

A Friesian-Brown Swiss steer, 9 months old, appeared ill with hyperaesthesia over the hindquarters and mild ataxia on 31 January, but maintained appetite until becoming permanently recumbent on 7 February when he was killed humanely. No necropsy was done.

A Guernsey cow, 6 years old, and a Friesian-cross cow, 2 years old, both developed moderately reduced milk production, posterior ataxia with exaggerated lifting of hind limbs, spasmodic shutting of eyelids, muscle tremors in flank and muzzle and drooling of saliva on 1 February, but maintained appetite. Two yearling heifers had mild stiffness of gait at this time as well. These 4 cattle were still mildly affected on 12 March.

Case 4 (Contributing authors RAM, RGS)
Six steers of body weight 340 to 380 kg in a herd of 80 near Eidsvold fed sprouted barley grain produced hydroponically in a

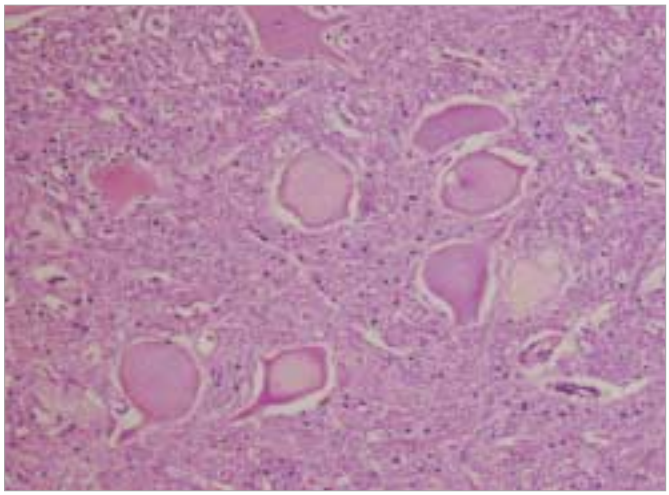

Figure 2. Central chromatolysis, cytoplasmic vacuolation and necrosis of neurones in a midbrain nucleus (Case 3). Haematoxylin-eosin x 80.

'fodder factory' system developed stiff gaits and their rate of weight gain decreased beginning on 27 February 2003. Access to the material was stopped immediately. The system had been operating for 49 days. A blue mould growth was first noted around the grain on 17 February, one week after an air blower failed, causing increased temperature and humidity in the facility. A sample of sprouted grain was confirmed as infected with *A clavatus*. A specimen was deposited in the Department of Primary Industries Plant Pathology culture collection as accession BRIP39339a. The sprout production system was thoroughly cleaned and disinfected with 125 mL sodium hypochlorite (Domestos Hospital Strength Bleach®) in 5 L water applied to the trays of grain at the beginning of the growth cycle and the running temperature of the system was reduced to a mean 22°C, but *A clavatus* infestation occurred in new batches of sprouted grain. By 12 March, the affected steers again appeared normal, but one had mild ataxia with knuckling of hind fetlocks when the herd was mustered on 22 March. This steer had recovered by 2 April, 32 days after the last intake of mouldy sprouted grain. No deaths occurred and no necropsies were done. The owner subsequently treated the water supply to his sprout production facility with copper sulphate at 220 mg /L (5 kg in 22,000 L) to try to prevent *A clavatus* growth. This aim appeared to be achieved, but serious stunting of sprout root growth occurred.

Discussion

Clinical and pathological findings in our cases were consistent with descriptions of *A clavatus* toxicity of ruminants elsewhere in the world. Signs common to our cases and those reported elsewhere were retention of appetite, posterior ataxia, knuckling of fetlocks, dragging of hind legs, high stepping with the hind limbs, stiff gait, tremors, progressive paresis, hypersensitivity, loss of milk production, recumbency, and clonic convulsions.[4-11] Signs reported elsewhere but not detected in our cases were a mild febrile reaction, frothing at the mouth, anorexia, ruminal stasis, constipation, dog sitting, urinary incontinence, the abnormal behaviours of pawing or tapping the ground with a foreleg, or marked raising of forefeet as if in pain, aggression, and paralysis in lateral recumbency.[4-11]

Necropsy lesions reported elsewhere in cattle but not detected in our cases were swelling, haemorrhage and pale skeletal muscles with oedema of intermuscular fasciae, and pale foci in the heart muscle.[4,11] Histological lesions reported have been degeneration and necrosis of large neurones in nuclei of the brainstem, the ventral horns of the spinal cord, and in spinal ganglia.[4-6,8-11] Neurones in the cerebral and cerebellar cortices were unaffected.[4,11] Changes reported in affected neurones have been swelling, central or complete chromatolysis, shrunken, pyknotic, lysed or absent nuclei, eccentrically displaced nuclei, and cytoplasmic vacuolation (more common in cells without visible nuclei).[4,5,9-11] Wallerian degeneration of spinal cord white matter tracts, fatty change in hepatocytes and hyaline degeneration of skeletal muscle fibres have also been reported in some cases.[4,6,8-11] Not all lesions occurred in each affected animal. On the other hand, no such lesions have been reported in botulism, bovine spongiform encephalopathy or other tremorgenic or convulsive syndromes of ruminants such as poisoning by lolitrems (perennial ryegrass staggers), corynetoxins (annual ryegrass toxicity, floodplain staggers), paspalitrems (*Paspalum* staggers) and indole alkaloids of *Phalaris* spp.[16]

A syndrome in late pregnant and recently-calved heifers fed mouldy sprouted barley grain from 'fodder factories' in Western Australia appears to differ from our cases in having very high serum glutamate dehydrogenase activity (up to 500 IU/L) and γ-glutamyltransferase activity (up to 1000 IU/L) (JG Allen, personal communication) 3 to 4 weeks after sprouted barley feeding stopped, but no liver lesions in necropsy material. *Aspergillus* spp were cultured from the barley sprouts. *Pyrenophora semeniperda,* common on barley grain in Western Australia in 2002, was believed to be capable of producing mycotoxins that could produce a similar syndrome (JG Allen, J Creeper, R Graydon, unpublished data, 2002). Hepatic cirrhosis, hepatocyte degeneration and nephrosis were reported in dairy cows fed 3 kg each daily of sprouted barley grain infested with *A clavatus* in Brazil[17] and necrosis of hepatocytes was reported in cows with the tremorgenic syndrome in China.[7]

Ruminants have been poisoned experimentally and the syndrome reproduced by dosing *A clavatus.* Sorghum beer residues naturally infested by *A clavatus*, when dosed to a calf (total dose 40 g/kg over 5 days) and a sheep (total dose 60 g/kg over 8 days), produced the tremorgenic syndrome, as did a pure culture of *A clavatus* dosed to a yearling steer (total dose 85.5 g/kg over 2 days).[4] Maize sprouts naturally infested with *A clavatus* dosed to a steer (8 g/kg daily for 16 days to onset of signs) and to a sheep (10 g/kg daily for 12 days to onset of signs) reproduced the tremorgenic syndrome, as did a pure culture of *A clavatus* inoculated into sterilised maize and dosed to a sheep (7.5 g/kg daily for 13 days).[5] Beer residues infested with *A clavatus* fed to a sheep at 37 g/kg daily for 35 days produced no clinical signs, but neuronal lesions were detected in spinal cord grey matter.[11] A number of toxins have been isolated from *A clavatus*, but none has been tested in ruminants to determine responsibility for the tremorgenic syndrome. Cytochalasin E killed rats within 2 to 18 h of dosing, causing cerebral oedema, ascites and lung haemorrhage.[18] Tryptoquivaline and tryptoquivalone injected intraperitoneally in rats each produced fine tremors and hypersensitivity persisting for 5 days after dosing, but no lesions at necropsy or microscopic examination of tissues, including brain.[18] Patulin extracted from culture filtrates of *A clavatus* in France and injected intraperitoneally into mice has produced signs consistent with those seen in cattle, but neuronal degeneration in only the cerebral cortex.[19] The most toxic fraction extracted from sorghum beer residues in South Africa and capable of producing the tremorgenic syndrome in sheep dosed orally contained neither patulin, tryptoquivalone nor nortrypto-

quivalone.[4] So the identity of the toxin or toxins responsible for the ruminant tremorgenic syndrome is yet to be determined.

In most cases described in other countries[5,9] no particular events that could have promoted fungus growth on the sprouting grains were linked with the onset of intoxication. In one case[10] a heat wave occurred several weeks before the incident, with ambient temperatures above 40°C for several days. In some of these cases[5, 10] the sprouted grain had been fed without ill effect for long periods before the toxicity incidents occurred. This is consistent with our cases 2 and 3. The equipment malfunction in the 'fodder factories' in our cases 1 and 4, leading to increased air temperatures, appears to be related to the subsequent *A clavatus* growth and intoxication. *A clavatus* growth is significantly promoted by a temperature rise from below 20°C to 25°C during the malting (germination) of barley, and may cause a 10,000-fold increase in spore counts.[20] *A clavatus* optimum growth temperature is between 20 and 35°C [1,21,22] with maximum growth and sporulation occurring at pH 6.5 to 7.5.[1,22] The minimum relative humidity for *A clavatus* growth has been measured as 88%, with optimum growth at 93 to 98%.[1] These conditions were broadly met in the production facilities in our cases.

Managing this intoxication has not been thoroughly addressed in the veterinary literature. *A clavatus* is difficult to eradicate from commercial maltings, predominating over other fungi when temperatures exceed 25°C.[20] So, preventing or controlling *A clavatus* infestations in grain sprouting facilities in summer in northern Australia is challenging. The strict, consistent use of good hygiene and running 'fodder factories' at the lowest temperature compatible with economic production, aiming at below 20°C, are fundamental. Cobalt in concentrations of 350 or 500 mg/mL inhibit *A clavatus* growth in liquid media[23] and crushed *Origanum majora* (marjoram, oregano) leaves inhibit *A clavatus* spore germination,[24] but neither has been applied under field conditions. Hypochlorite treatment of malt barley has not been effective for the control of *A clavatus* infestations and may actually favour the fungus by reducing populations of competing species.[1] The owners of cattle in cases 2 and 4 both had poor results from using hypochlorite treatment in their sprout production facilities. Another cattle owner feeding sprouted barley grain from a 'fodder factory' reported effective control of *A clavatus* infestations by adding wettable sulphur to the hydroponic water supply at 148 mg/L (4 kg added to a 27,000 L tank) (RG Shivas, unpublished). Sprouted grain so produced contained 2500 mg sulphur/kg, 5.7 mg copper/kg and 1.74 mg molybdenum/kg (GW Wöbke, personal communication). This sprouted grain was unlikely to cause sulphur-associated polioencephalomalacia, but may lead to copper deficiency in cattle.[25]

Feeding sprouted grains to ruminants has recently become more common in Australia. Contributing reasons included widespread drought conditions during 2002-3 causing feed shortages, and a belief that nutritional advantages may accrue from feeding grain as sprouts. Studies of the nutritional value of hydroponically sprouted grains for ruminants do not support the latter view.[26, 27] The cost of barley sprouts has been calculated as about $1/kg dry matter, which compares poorly with grain at $0.3/kg dry matter (RJ Sneath, personal communication). *A clavatus* neurotoxicity has occurred sporadically in many places in the world where sprouted grains have been fed to cattle. Our cases confirm that Australia is not exempt from this pattern and that any advantage gained from this practice can be heavily outweighed by the largely unpredictable risks of poisoning in ruminants and of extrinsic allergic alveolitis in humans exposed to *A clavatus* spores.

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