Onderstepoort J. vet. Res., 43 (3) 147–154 (1976)

A HIGHLY FATAL TREMORGENIC MYCOTOXICOSIS OF CATTLE CAUSED BY *ASPERGILLUS CLAVATUS**

T. S. KELLERMAN(1), J. G. PIENAAR(1), G. C. A. VAN DER WESTHUIZEN(2), L. A. P. ANDERSON(1) and T. W. NAUDÉ(1)

ABSTRACT

KELLERMAN, T. S., PIENAAR, J. G., VAN DER WESTHUIZEN, G. C. A., ANDERSON, L. A. P. & NAUDÉ, T. W., (1976). A highly fatal tremorgenic mycotoxicosis of cattle caused by *Aspergillus clavatus*, *Onderstepoort Journal of Veterinary Research*, 43 (3) 147–154 (1976)

During February 1975, a tremorgenic neurotoxicosis decimated a herd of cattle in the northern Transvaal. This hitherto unidentified disease was characterized by hypersensitivity, incoordination, a peculiar stiff-legged gait of the hind legs, severe generalized tremors of the skeletal muscles, progressive paresis, paralysis and constipation.

The most notable gross pathological lesions were degenerative and necrotic changes in certain skeletal muscles, haemorrhages on the serosal surfaces, especially on the dorsal aspect of the rumen, and gastro-intestinal stasis. Microscopical examination of the central nervous system revealed cytopathological changes consisting of degeneration and necrosis of the large motor cells in the ventral horns of the spinal cord and bigger neurones in numerous nuclei of the medulla oblongata, midbrain and thalamus.

By feeding the suspect ration and its component parts to cattle and sheep, it was possible to identify mouldy sorghum beer residue as the toxic component in the ration. *A. clavatus*, the dominant fungus on the toxic residue, was readily isolated in pure culture. The entire syndrome was then reproduced in a yearling Friesland steer dosed with pure cultures of the *A. clavatus* isolate grown on autoclaved non-toxic sorghum beer residue. The toxic principle is not known, but it does not appear to be patulin, tryptoquivalone, tryptoquivaline, or any other known tremorgen.

Résumé

UNE MYCOTOXICOSE TRÉMORGÈNE MORTELLE À ASPERGILLUS CLAVATUS *CHEZ LE BOVIN*

Au mois de fevrier 1975, *un troupeau de bovins au Transvaal du nord a été abattu par une neurotoxine trémorgène. Les symptômes caractéristiques de cette maladie, identifiée pour la première fois, ont été l'incoordination, une allure curieuse aux pattes postérieures rigides, trémulations excéssives et généralisées des muscles striés, la parésie, la paralysie et la constipation.*

Les lésions macroscopiques signalées ont été la dégénérescence et la nécrose de certains muscles striés, des hémorragies des séreuses surtout au niveau de la paroi dorsale de la panse et une stase gastro-intestinale. Les coups histologiques du névraxe ont mis en évidence la dégénérescence et la nécrose des cellules motrices des cornes ventrales de la moelle épinière ainsi des neurones au niveau de nombreuses noyaux du bulbe et du thalamus.

*En donnant à manger aux bovins et moutons la ration suspecte, les auteurs ont pu identifier comme composant toxique les résidus de sorgho utilisés dans le brassage de la bière. Ce résidu était substrat aux moisissures, dont on a pu avec facilité isoler en culture l'*A. clavatus *comme moisissure principale. Ensuite les auteurs ont pu reproduire la maladie en injectant* per os *un taurillon âgé d'un an avec une culture pure d'*A. clavatus *cultivée sur résidu de sorgho autoclavée et non-toxique. On ignore encore la substance toxique, mais elle ne parait être ni la patuline, ni la tryptoquivalone, ni la tryptoquivaline, ni aucune autre substance trémorgène connue.*

INTRODUCTION

Considerable attention has been paid in recent years to the tremorgenic metabolites of *Penicillium* and *Aspergillus* spp., with the result that many tremorgens have come to light. The known tremorgenic metabolites of species of the genus *Penicillium* include an intracellular tremorgen ($C_{37}H_{44}NO_6Cl$) isolated from *P. cyclopium* (Wilson, Wilson & Hayes, 1968). This tremorgen, named tremortin A, together with a chemically-related compound, tremortin B ($C_{37}H_{45}NO_5$), was subsequently recovered also from *P. palitans* (Ciegler, 1969; Hou, Ciegler & Hesseltine, 1970). The production of tremortins was not limited to only these 2 species, however, (Ciegler & Pitt, 1970); 3 other species of the genus *Penicillium*, namely *P. crustosum*, *P. granulosum*, and *P. puberculum* (of the subsection Fasciculata, section Asymmetrica) were also found to possess this property (Ciegler & Pitt, 1970; Hou, Ciegler & Hesseltine, 1971). A 3rd tremorgen, verruculogen ($C_{27}H_{33}NO_7$) was obtained from *P. verruculosum* (Cole, Kirksey, Moore, Blakenship, Diener & Davis, 1972; Fayos, Lokensgard, Clardy, Cole & Kirksey, 1974), and recently Cole, Kirksey & Wells (1974) reported the isolation of yet another tremorgen ($C_{27}H_{33}NO_4$) from *P. paxilli*. The first tremorgenic metabolite from the genus *Aspergillus* was isolated from *A. flavus* by Wilson & Wilson (1964). This was followed by the isolation of 2 others, trivially known as fumitremorgen A ($C_{33}H_{45}O_6N_3$) and B ($C_{26}H_{29}O_6N_3$) from *A. fumigatus* (Yamazaki, Suzuki & Miyaki, 1971), which appear to be chemically related to the tremorgen from *P. paxilli* and to verruculogen, as they have a 6-0-indole moiety in common (Fayos *et al.*, 1974). Verruculogen and fumitremorgen have also been isolated from *A. caespitosus* (Fayos *et al.*, 1974).

In this study it will be shown that *A. clavatus*, growing on sorghum beer residue, produced an unknown tremorgenic metabolite(s) which caused severe mortality of cattle on a farm in the northern Transvaal. This species is known to form mycotoxins such as patulin (Bergel, Morrison, Moss, Klein, Rinderknecht & Ward, 1943), cytochalosin E (Glinsukon, Yuan, Wightman, Kitaura, Büchi, Shank, Wogan & Christensen, 1974), escladiol (Suzuki, Takeda & Tanabe, 1971) and 2 tremorgenic substances, tryptoquivalone and tryptoquivaline (Glinsukon *et al.*, 1974; Clardy, Springer, Büchi, Matsuo & Wightman, 1975). *A. clavatus* has, furthermore, been associated with nervous disorders of cattle in France.

(1) Veterinary Research Institute, Onderstepoort 0110
(2) Plant Protection Research Institute, Private Bag X134, Pretoria, 0001
* Paper presented at the Biennial National Congress, South African Veterinary Association, Durban, Sept. 1975

Received 1 July 1976—Editor

In one outbreak it was the dominant fungus on toxic sprouted wheat fed to dairy cattle. Affected animals developed incoordination, often fell down on smooth surfaces, walked with a high stepping gait of the hind legs, were very nervous, and suffered from muscular tremors. Some apparently normal animals lay down and remained recumbent until death. These signs were accompanied by a mild febrile reaction, anorexia, ruminal stasis, constipation and a drop in milk production (Moreau & Moreau, 1960). In another outbreak cattle developed nervous signs also after eating germinated wheat predominantly infected with *A. clavatus* (Jacquet, Boutibonnes & Cicile, 1963). The animals were sometimes aggressive, resentful of contact, and restless. At first they walked about incessantly and unsteadily, but later became recumbent. In an effort to establish the cause of the intoxication described by Moreau & Moreau (1960), Capitaine & Bolouet (1974) administered patulin by various routes to mice. The mice that were injected via the intraperitoneal route developed symptoms similar to those of the cattle. These symptoms were attributed to neuronal degeneration in the cerebral cortex.

Natural outbreaks of tremorgenic conditions are very rare. A notable exception, however, is Bermuda grass tremors (BGT) of cattle in the United States. This disease (Diener, Davis, Morgan-Jones, Cunningham, Langford & Johnson, 1975) afflicted 25 000 cattle in over 500 herds in Louisiana in 1971, and resulted in the death of 600. Affected cattle become excitable, twitch, tremble, show incoordination, become stiff in the hind quarters, and may fall down, rising again only with difficulty. They remain alert but quickly lose condition, milk production drops, and some may abort. Death usually ensues from secondary effects such as pneumonia and self-trauma. Recent work has indicated that alkaloids produced by a *Claviceps* sp. isolated from toxic Bermuda grass may be implicated in the aetiology of the disease (Porter, Bacon & Robbins, 1974).

Description of the Field Outbreak

In February 1975, a catastrophic nervous disease broke out amongst cattle on a farm in the northern Transvaal. Within a month 130 out of a herd of 330 died of the disease, 70 carcasses were condemned at the abattoir, and 60 head suffered irreversible brain damage.

Farming practices

The disease broke out in 3 herds composed, respectively, of 232 oxen (aged 18 months–2 years), 76 cows, and 22 young bulls. The affected cattle grazed on natural summer pastures and had free access to a supplementary ration consisting of citrus meal (28,6%), sorghum beer residue (57,1%) and maize meal (14,3%). About 10 kg of freshly made-up ration was eaten per head per day.

Clinical signs

The disease was characterized by hypersensitivity, severe muscular tremors, ataxia, progressive paresis, paralysis, and constipation. The tremors, which usually started inconspicuously in the flanks, spread and soon affected almost the entire body. Affected animals had a peculiar stiff-legged gait, holding the hind quarters very upright, taking short steps and sometimes knuckling over. Upon being startled, they would assume a splaylegged stance with the hocks slightly bent and forelimbs and neck held rigidly extended, while severe muscular spasms shook their bodies. Such animals frequently staggered drunkenly about and fell down on their sides, or pitched forward onto their sterna. In time, most of the affected cattle became recumbent. At first they rested in a normal sternal position, but, as the paresis progressed, they fell onto their sides, lying thus paralysed and trembling, until they died.

Apart from a tendency to over-react to outside stimuli, stricken animals behaved fairly normally. They were apparently well aware of their environment, and would turn their heads or prick up their ears in response to sounds or movements that attracted their attention.

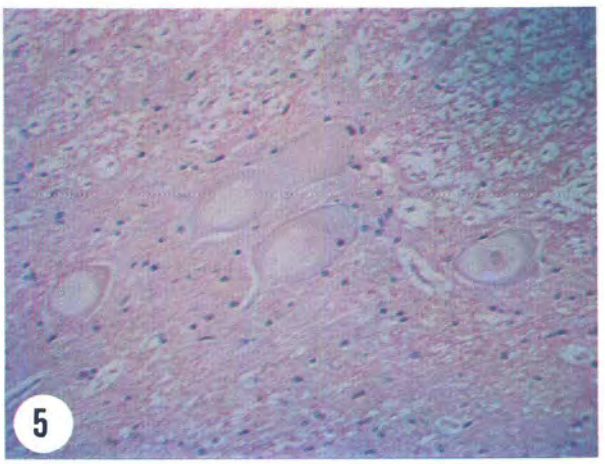
Although the tongue, limbs, and tail were seldom completely paralysed (cf., botulism), many animals could not eat or drink. In such cases saliva dribbled from the mouth and signs of dehydration were present. Ruminal movements often persisted to a fairly late stage of paresis but chronically affected animals were invariably found to be constipated. The condition was afebrile and death usually occurred within 2–7 days of the onset of signs. Those that survived sometimes displayed lasting locomotory disturbances, such as clumsiness and a stiff-legged gait, which were often accompanied by swelling of certain joints of 1 or more legs, probably due to self-trauma as a result of ataxia.

Gross pathology

In those animals that were killed or that died shortly after the onset of signs, the gross pathological lesions were not as marked as in the cases that showed clinical signs for longer periods.

Marked *rigor mortis* of the limbs occurred within minutes of death, both in animals dying naturally and in those that were slaughtered. The most obvious gross lesions were seen in the skeletal musculature, which showed degenerative and necrotic changes. Whole muscles, or, more commonly, focal areas of muscles, particularly near their insertions or origins, had a swollen appearance with a greyish-white discoloration (Fig. 4). Petechial haemorrhages frequently occurred in the affected parts of the muscles. The muscles most commonly affected were: the gastrocnemius (particularly the deep head), semimembranosus, semitendinosus, quadriceps femoris group, serratus ventralis, gracilis, ilio-psoas, sternal muscles and the extensor muscles of the front limbs. Oedema of the intermuscular fasciae and scattered petechial haemorrhages (most prominent along the course of larger nerves, e.g. ischiatic and brachial) were present in most cases. Petechiae and ecchymoses were also scattered over most of the serous surfaces and large paint brush haemorrhages were seen on the dorsal serosal surface of the rumen in some of the more acute cases. Subepicardial and subendocardial haemorrhages occurred commonly in varying degrees of severity.

The myocardium as a whole often had a "parboiled" appearance, and in cases that survived for longer periods, small focal areas of necrosis were frequently present in papillary muscles and the wall of the left ventricle. Mild oedema of the lungs was sometimes seen. In the gastro-intestinal tract the most common changes were: stasis of the fore stomachs, hyperaemia and petechial haemorrhages in the caecum, and constipation.



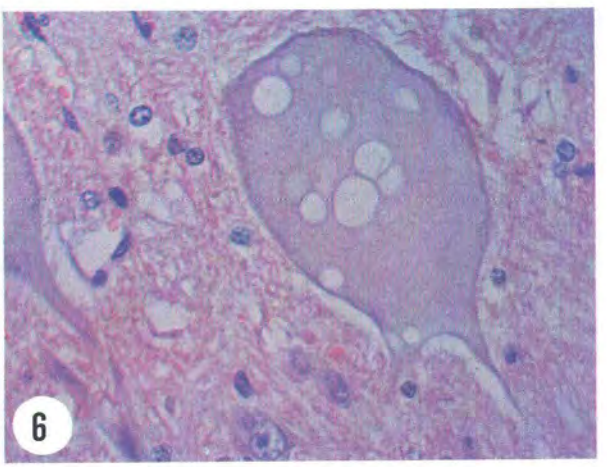


FIG. 1 *Aspergillus clavatus* cultured on sorghum beer residue
FIG. 2 Affected animals have a stilted gait, knuckle over and tremble
FIG. 3 Terminally animals become paralyzed
FIG. 4 Pronounced muscular degeneration and necrosis
FIG. 5 *Medulla oblongata*. Group of neurones showing swelling, chromatolysis, and loss of nuclei. HE×200
FIG. 6 A vacuolated neurone. HE×560

Animals killed shortly after the onset of signs showed marked congestion of the meningeal vessels, while, in the case of animals that died or were slaughtered after a few days, no remarkable changes were noticed. In practically all cases, the synovial fluid in the joints had a turbid dark, straw-coloured appearance. No lesions could be detected, however, in the synovial membranes.

Microscopic pathology

The specimens were collected and prepared as described for the experimental cases (see: Feeding trials with suspect ration). Cytopathological changes, consisting of degeneration and necrosis of neurones, were the most significant microscopic lesions seen. The large motor cells of the ventral horns of the spinal cord and the larger neurones in numerous nuclei of the medulla oblongata, midbrain and thalamus were mainly affected, but neurones in the cerebral and cerebellar cortices were not involved.

Affected cells in animals that died shortly after the onset of the outbreak showed a dense, slightly eosinophilic cytoplasm, shrunken pyknotic nuclei and enlarged perineuronal spaces. Many of these cells contained no nuclei, while in others nuclear fragments or faded silhouettes of nuclei were visible. Fine vacuoles were also scattered in the cytoplasm of these cells, and sometimes they were concentrated more closely around the periphery of the cytoplasm.

More striking changes were seen in the neurones of animals that showed clinical signs for a few days before they died or were slaughtered. Marked swelling of the cells occurred, giving their contours a rounded appearance. Owing to complete chromatolysis, the cytoplasm of these cells appeared to be finely granular and markedly eosinophilic (Fig. 5). Some cells still showed a narrow zone of Nissl granules near their margins. Eccentrically situated nuclei lying against the cell membrane were frequently present. These were frequently oval or reniform in shape, and swollen, taking on a hypochromatic appearance, while their nucleoli were enlarged and stained deeply basophilic. Fragmentation and fading of nuclei were commonly seen in these enlarged eosinophilic neurones and, in many, complete disappearance of the nuclei had taken place. Vacuoles of varying size, seen in the cytoplasm of some of the enlarged eosinophilic neurones (Fig. 6), were more common in cells where the nuclei were no longer visible.

Microcavitation, seen as vacuoles of varying size, was present in the white substance around some of the nuclei containing affected neurones. This microcavitation also occurred irregularly in the white substance of the spinal cord, most commonly in the immediate surroundings of the ventral horns. In some of the cavities, structures resembling disintegrated axis cylinders were seen. Satellitosis and neuronophagia were not prominent features and were observed only infrequently. Focal gliosis, where neurones had disappeared, was seen in some of the more chronic cases.

The affected skeletal muscles showed typical hyaline degeneration and necrosis.

Feeding trails with the Suspected Ration

Methods

Since only those cattle that had access to supplementary feed were affected, and all other likely causes, such as plant or pesticide poisoning, had been excluded, it was decided to investigate the possibility that the ration was toxic.

The suspect ration and its various component parts were separately dosed to cattle and sheep as summarized in Table 1. All the animals were dosed per stomach tube, with the exception of Bovine 1, Bovine 3 and Bovine 6, which voluntarily ate the material. At times the continuity of the dosing programmes was interrupted, e.g. over week-ends or when the test animals developed digestive disturbances.

The animals were examined each day and periodically the following routine chemical pathological determinations were done on the blood: gamma glutamyl transpeptidase (γ–GT); glutamic oxaloacetic transaminase (GOT); total plasma proteins; glucose; serum urea nitrogen; and serum calcium, sodium, and potassium.

Animals that reacted positively were either allowed to die naturally (Bovine 1 and Sheep 1) or were destroyed by the intravenous injection of pentobarbital sodium* (Bovine 2, Bovine 3, Bovine 4, and Sheep 2). Specimens from various organs were taken at autopsy, fixed in 10% buffered formalin, sectioned in a routine manner, and stained with haematoxylin and eosin (HE). The brains and spinal cords were fixed *in toto* in approximately 30% buffered formalin before sectioning.

Results

Clinical Signs

1. *Complete ration*

Bovine 1 was dosed 7 times in 8 days, initially with 5 and later with 10 g/kg, of the complete ration (Table 1). Apart from mild digestive disturbances due to overfeeding, nothing untoward happened until Day 6, when the young bull trembled slightly while walking the short distance from the crush pen to its stall. It also appeared to be confused, took wrong turnings along the route, and once fell down while negotiating a shallow drainage channel. Later it became markedly ataxic, developed a peculiar stiff-legged gait, and frequently fell while turning around or walking over uneven surfaces. A pronounced tremor of the muscles was evident in the hind quarters and flanks, the animal was weak and salivated profusely. It was also hypersensitive to both auditory and visual stimuli. Late on Day 6 it became permanently recumbent after a fall. Thereafter the paresis gradually worsened until the animal was almost completely paralysed. After Day 9 it refused to eat or drink, and, although the tongue was never markedly paretic, a pool of saliva formed in front of it. Passing a stomach tube now became difficult, indicating that the oesophagus was probably paralysed. The animal was found dead in its stall on the morning of Day 10.

Bovine 2 refused to eat the ration unless it was diluted with maize meal. Between 2,5 and 5 g/kg of the ration mixed with equivalent amounts of maize meal was therefore fed to it over a period of 9 days (Table 1). The first clinical signs, namely, mild hypersensitivity and tremors of the muscles of the hind quarter, appeared after light exercise on Day 8. By the following day almost all the muscles trembled and the animal walked with a typical stiff-legged gait. It was destroyed for autopsy early on the 10th day.

* Maybaker

TABLE 1 Administration of the suspected poisonous ration to cattle and sheep

Material administered	Test animal	Sex M=male F=female C=castrated	Age (permanent incisor teeth)	Initial live mass (kg)	Dosing regimen (g/kg/day) ×n*	Total mass dosed (kg)	Duration of experiment (days)	Result
Complete ration.....	Bovine 1	M	2	301	10×3 5×4	16,0	10	Tremorgenic syndrome
Complete ration.....	Bovine 2	C	2	214	2,5×2 5,0×7	8,6	10	Tremorgenic syndrome
Complete ration.....	Sheep 1..	C	8	45	10×5 20×5	6,7	13	Tremorgenic syndrome
Sorghum beer residue..	Bovine 3	F	8	385	5×7	13,5	17	Hepatotoxicosis
Sorghum beer residue..	Bovine 4	M	0	217	10×3 5×2	8,7	7	Tremorgenic syndrome
Sorghum beer residue..	Sheep 2..	C	8	35	10×6	2,1	13	Tremorgenic syndrome
Citrus meal.....	Bovine 5	M	0	180	10×2 5×2 2,5×4	8,9	18	Negative
Maize meal.....	Bovine 6	F	8	547	5×13	35,5	17	Negative

* n=number of doses given

Sheep 1 was dosed 10 times with 10–20 g/kg of the ration over a period of 12 days (Table 1). The first clinical signs appeared on Day 7, and early on Day 13 it was found dead in its stall. During the course of the disease, the sheep became hypersensitive, all the muscles of the body appeared to tremble, and progressive paresis set in. Terminally the sheep lay semi-paralysed, trembling, and gasping for breath.

2. *Sorghum beer residue*

Bovine 3, an 8-year-old Jersey cow, was given 5 g/kg/day of the residue for 7 days (Table 1) and kept under observation until euthanized on Day 17. Although the cow became slightly lame in the hind legs (probably because of the high energy diet), had a slightly swollen vulva and trembled slightly after exercise, it never developed typical signs of the disease.

No chemical pathological abnormalities were noticed on the 6 occasions on which the animal was bled before Day 10, but after that a marked elevation of γ–GT activity occurred. The values recorded on Days 10, 13, 16 and 17 were respectively 21, 112, 650 and 1 350 mIU/ml. The rise in γ–GT activity was accompanied from Day 16 onwards by an elevation of GOT activity (serum level, 247 King Units), mild bilirubinaemia (total bilirubin, 1,6 mg/100 ml), and haemoconcentration (red blood cell volume, 86%). Similar changes suggestive of liver damage were not encountered in any of the other cases.

Bovine 4 was dosed with 8,7 kg of residue divided into 5 doses of 5 or 10 g/kg over 5 days (Table 1). Typical clinical signs were seen from Day 4-Day 6. The calf suffered from progressive paresis and showed other signs of the disease, such as incoordination, a stiff-legged gait, muscular tremors, salivation, and hypersensitivity. Upon the slightest provocation it would shake violently and assume the splay-legged stance described previously. During such attacks it often tottered about on stiff legs and fell down. At other times it also fell down because of lack of co-ordination while negotiating rough ground. In both instances the hind limbs were frequently held in unnatural positions while the animal was down. On Day 6, when it could no longer stand, eat or drink, it was destroyed for autopsy.

Sheep 2 received 6 doses of 10 g/kg of the residue over a period of 8 days (Table 1). Typical signs were seen from Day 5 until the sheep was destroyed for autopsy on Day 13. During this period muscular tremors (starting in the hind quarters), aberrations in gait (see Sheep 1), hypersensitivity and paresis/paralysis were evident.

3. *Maize meal and citrus meal*

The above components of the ration produced no ill effects at dosage rates approximately equivalent to the other toxic materials (Table 1).

Sorghum beer residue could therefore be identified as the toxic element in the ration.

Pathological lesions

The gross and microscopic pathology of the experimental cases concurred with that of the natural ones, differing only in the severity of the lesions. Generally speaking, the pathological changes in the neurones were more marked and larger numbers of neurones were affected in the experimental cases. In the experimental sheep, degeneration and necrosis of muscles were either absent or very mild. Bovine 3 differed from all other cases in that, in addition to typical changes in the central nervous system, it showed hepatopathy. This was reflected in icterus of the carcass accompanied by hepatomegaly and focal areas of necrosis throughout the liver. Fatty changes were seen around the central veins. Small foci of haemorrhage and liquefactive necrosis with mild neutrophile infiltration were scattered irregularly throughout the liver. In the portal tracts, a mixed round cell-polymorphonuclear cellular infiltration was present. Obvious oedema and moderate fibroplasia occurred around the portal tracts and proliferation of bile duct epithelium was evident. The layers of connective tissue were concentrically arranged around some of the larger bile ducts.

Mycotoxicological Investigation of the Sorghum Beer Residue

General observations pertaining to the natural outbreak

Procedure for handling sorghum beer residue: Each consignment was collected at the brewery on the day of manufacture (Fig. 7), and spread out in a thin layer (*c.* 3–5 cm deep) on the floor of a shed at the farm. During the period that it lay there (maximum 3 days) before being incorporated into the ration, it was regularly turned over with shovels.

Climatic conditions before the outbreak: Heavy rains fell in the month before the outbreak (Fig. 7) and diurnal temperatures were high. At 16h00 on a typical February afternoon the temperature was 31 °C.

Conditions at the brewery: The residue is a by-product of sorghum beer, which is manufactured under strictly controlled hygienic conditions. Examination of a typical batch of residue at the brewery did not reveal visible mould growth nor could many fungi be isolated from it by means of routine techniques. The relative absence of fungi can probably be ascribed partly to the effects of fermentation and partly to the application of heat under slight pressure (*c.* 106 °C and *c.* 40 kPa) during the brewing process.

Methods

Isolation of the toxic fungus: Particles of toxic sorghum beer residue were incubated at 26 °C on 1,5% malt extract agar (MEA) plates containing sodium novobiocin* (100 mg/l). The dominant fungus, namely *Aspergillus clavatus* (Fig. 8), was isolated in pure culture.

Inoculum for the bulk cultures was prepared by transferring spores of these pure isolates onto the surface of MEA contained in 250 ml flasks (100 ml/flask), and incubating the material for 7 days at 26 °C in the dark. The spores were harvested in distilled water containing 0,25% Agapon** (200 ml/flask).

Preparation of bulk cultures and control material for dosing trials: A large quantity of fresh sorghum beer residue was autoclaved for 2 h at 121 °C and 1.03 bar in 1 litre flasks (*c.* 400 g/flask). Some of the flasks were inoculated with the spore suspension (5 ml/flask) and incubated together with the uninoculated ones (Control 1) for 6–7 days at 26 °C in the dark. As an additional control, a quantity of the fresh residue was stored in a freezer (0 °C) for 24 days and autoclaved as described above (Control 2).

* Upjohn
** Agfa-Gevaert

Dosing trials: The pure culture of *A. clavatus* (Fig. 1) and the uninoculated sorghum beer residue substrate were dosed per stomach tube to animals as shown in Table 2. Otherwise, the trial was conducted in a manner similar to that of the suspect ration.

Results

Clinical signs

Bovine 7, a yearling Friesland steer, was dosed with *c.* 43 g/kg of pure culture material on 2 consecutive days (Table 2). Twenty hours after the 1st dose was given the steer was seen to salivate slightly. This was followed by typical clinical signs at 44 h: viz., the animal was restless and hypersensitive; it walked with the characteristic stiff-legged gait; when startled it assumed the typical splay-legged stance with forelimbs and neck extended, while spasms shook its body; the muscles of the hind quarters, shoulders and neck trembled constantly (Fig. 2). Within 64 hours after the 1st dose was given, the steer was found lying on its side semi-paralyzed and trembling (Fig. 3). At 71 h it was euthanized *in extremis*.

Apart from a marked increase in the heart and respiratory rates, and a slight terminal elevation of GOT activity in the serum (206 King Units), no other notable chemical pathological changes were recorded. The condition was afebrile and ruminal motility was still normal 24 h before the animal was destroyed.

Sheep 3 *and Bovine* 8, the 2 animals that received the sterilized non-inoculated residue (Table 2), suffered no apparent ill-effects. A minor, unexplained elevation of GOT and γ–GT activity occurred in the serum of Sheep 3 but no marked clinical pathological changes were seen in Bovine 8. Both animals were discharged from the experiment in excellent health.

Pathological findings: The gross and microscopic lesions on Bovine 7 were identical to those of the field and other experimental cases. No lesions were encountered in the liver.

Chemical Investigation

Toxic sorghum beer residue (28 kg) from the affected farm was extracted 3 times with hot methanol. The combined extracts were evaporated to a syrup on a waterbath under reduced pressure, and, when administered orally to a sheep at a dose of 1,5 g/kg live mass, caused typical tremorgenic symptoms within 72 h.

Chromatography of the toxic syrup on silica gel, using chloroform → methanol as eluent, yielded fractions which produced typical symptoms at dosage levels of 135 mg/kg, when dosed orally to a sheep.

TABLE 2 Administration of pure *A. clavatus* cultures and sorghum beer residue substrate to animals

Material administered	Test Animal	Sex C=castrated	Age (permanent incisor teeth)	Initial live mass (kg)	Dosing regimen (g/kg/day) ×n*	Total mass dosed (kg)	Duration of experiment (days)	Result
Pure culture.....	Bovine 7	C	0	221	*c.*43×2	18,9	3	Tremorgenic syndrome
Control 1.....	Sheep 3..	C	2	31	10×8	2,5	15	—
Control 2.....	Bovine 8	C	0	359	50×2	39,5	12	—

n*=Number of doses given

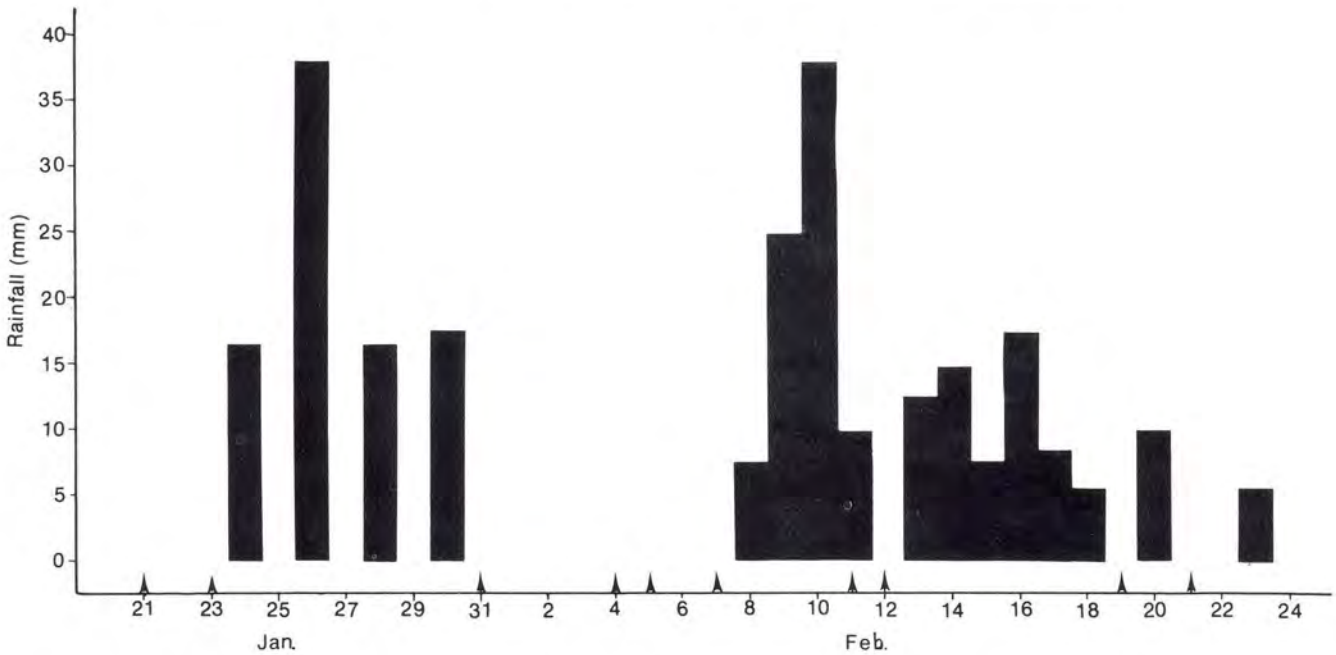


FIG. 7 Rainfall in the month prior to the outbreak, and dates* on which sorghum beer residue was collected at the factory

* ▲ date on which the residue was collected

FIG. 8 *Aspergillus clavatus* × 75

A silica gel thin layer chromatographic investigation of the most toxic fractions, using patulin*, tryptoquivalone* and nortryptoquivalone* as reference material, revealed the apparent absence of these toxins in our toxic material; nor could cyclopiazonic acid and tremortin A be demonstrated in the toxic residue (P. S. Steyn, National Chemical Research Laboratory, CSIR, Pretoria, 0002, personal communication, 1975).

Discussion

Sorghum beer is a very popular, mildly intoxicating drink which is consumed in large quantities by a considerable section of the population in southern Africa. Consequently, large amounts of residue are locally available, and, being very nutritious, it is much sought after as a supplementary feed for stock. It is fed both in the fresh wet form, and as a dry material.

Many questions regarding the circumstances leading up to the mycotoxicosis from the ingestion of this residue remain unresolved. For instance, one puzzling aspect of the outbreak was why only animals on this one farm were affected, seeing that sorghum beer residue is widely used in the area. Generally speaking, the incidence of a mycotoxicosis is dependent on 3 main requirements: a toxic fungus must be present, there must be a suitable substrate for it to grow on, and the meteorological conditions must be favourable for both growth and toxin production. On the non-affected farms, 1 or more of these factors must presumably have been absent. When the wide distribution of the fungus, the general availability of a suitable substrate and the uniformity of the weather conditions prevailing at the time are taken into account, it would appear that the only pertinent difference between conditions on the affected and non-affected farms lay in the manner in which the residue was handled. Investigations revealed that it was common practice in this area to stock-pile residue for days and weeks on end before it was used. Such heaps, being biologically active, would tend to heat up considerably at the core, and, at the same time, the outer surfaces would become so mouldy that they would have to be discarded. On the affected farm, however, very different conditions prevailed. Here the residue was not stock-piled for long periods but spread out on a floor for only a day or two. Spreading out the residue would have the effect of reducing microbiological activity by promoting desiccation. The mycopopulation would also be additionally inhibited by the short time available for the growth of fungi. Under such a system the residue can be expected to act as a selective medium for those fungi best able to survive under these adverse conditions, namely, fast growing, rapidly sporulating species such as *A. clavatus*. In the light of these arguments, and for other reasons too numerous to mention here, it can be seen that handling practices can have a profound effect on both the size and composition of the fungal populations of this commodity. In the case under review the very hygienic method of handling the residue could have helped to precipitate the mycotoxicosis by favouring the growth of the toxic fungus at the expense of its competitors. Another contributing factor may have been that, on the affected farm, all the residue was fed and nothing was discarded because of mould.

The explosive nature of the outbreak also needs to be explained. In this connection it can be postulated that *A. clavatus* spores gradually accumulated on the floor until the inoculum was large enough to transform the last batch of residue into a highly toxic, almost pure culture of the fungus. Alternatively, it may be argued that the gradual ingestion of small amounts of a cumulative toxin could produce a similar effect, but unfortunately it is not known at this stage whether the toxin is cumulative or not.

Another puzzling aspect of the mycotoxicosis is its apparent rarity. Only one outbreak has so far been recorded; yet the fungus is not uncommon and sorghum beer residue is widely used in summer when meteorological conditions favour the growth of mould. One explanation may be that minor outbreaks in the past have either gone undetected or undiagnosed. Alternatively, in some cases, erroneous diagnoses may have been made. *A. clavatus* poisoning can be confused with a number of other nervous disorders, notably botulism, but can easily be distinguished from them by the muscular tremors. No positive affirmative test for botulism in cattle is available and the diagnosis is often made on clinical grounds and by exclusion of other conditions. Control measures comprise the removal of the suspected poisonous feed and immunizing the animals. In retrospect, it would seem that several undocumented outbreaks of so-called atypical botulism which responded to the above measures might easily have been *A. clavatus* poisoning.

The disease that it most closely resembles, however, is "Licuari disease". During the autumns and winters of 1970, 1971 and 1974, this disease of unknown aetiology was responsible for the death of hundreds of cattle on 2 ranches bordering on the Licuari River near Quelimane in Mocambique (T. F. Adelaar & J. G. Pienaar, unpublished reports). The animals had grazed on natural pastures, and the first clinical signs noticed were excitability, wild staring eyes, and trembling of the muscles. Affected animals walked with a stiff, stilted gait, showed ataxia and went down, first on sternal and eventually on lateral recumbency. Paresis was followed by paralysis before the animals succumbed. In the early stages of the disease the animals showed muscular trembling, usually of the face, neck, shoulder and flank muscles, and ataxia when disturbed. In some of the animals, however, pronounced muscular spasms became evident over the whole body after excitement. Salivation and constipation were also common. Lesions in the skeletal muscles and heart, similar to those described for *A. clavatus* poisoning were seen in these cases. Other similarities in the gross pathology were hyperaemia of the abomasum and congestion of the brain in animals killed during the early stages of the disease. Microscopic examination of the neurones showed changes in the ventral horns of the spinal cord and nuclei of the medulla oblongata and midbrain closely resembling those of *A. clavatus* poisoning.

A. clavatus poisoning also bears some resemblance to the nervous disorders of cattle described by Moreau & Moreau (1960) and Jacquet *et al.* (1963) in France. In these outbreaks, *A. clavatus* was incriminated as the dominant fungus on the germinating wheat eaten by the poisoned cattle. Germinating wheat, like sorghum beer residue, is a predigested

* We wish to express our gratitude to Prof. G. H. Büchi of the Massachusetts Institute of Technology for samples of tryptoquivalone and nor-tryptoquivalone, and Dr W. F. O. Marasas of the National Research Institute for Nutritional Diseases for samples of patulin.

food. It would therefore be interesting to know whether a predigested substrate is necessary for the production of the toxin and whether the toxins involved in the outbreaks in France and South Africa were the same. Capitaine & Balouet (1974) induced neuronal lesions not unlike those described for cattle in this study by the intraperitoneal injection of patulin into mice. But the location of the lesions was different, and patulin could not be demonstrated in the residue that poisoned cattle in South Africa. It would appear, therefore, that, although patulin may have played a part in the outbreaks in France, this was not the case in South Africa.

The nature of the toxin can only be speculated upon, but from the evidence at hand it does not appear to be patulin, tryptoquivalone or tryptoquivaline. Efforts are now being made to isolate and characterize it.

Acknowledgements

We are grateful to our colleagues, Drr J. van Staden, G. H. Vogelsang and P. P. Bosman, for their whole-hearted assistance in carrying out this project. Special thanks are due to staff members of the Toxicology and Pathology Sections of this Institute, as well as to Miss Cicelia Roux of the Mycology Section, Plant Protection Research Institute, for invaluable technical assistance.

References

BERGEL, F., MORRISON, A. L., MOSS, A. R., KLEIN, R., RINDERKNECHT, H. & WARD, J. L., 1943. An antibacterial substance from *Aspergillus clavatus* and *Penicillium claviforme* and its probable identity with patulin. *Nature, London*, 152, 750.

CAPITAINE, R. & BALOUET, G., 1974. Étude histopathologique des lésions induites chez la souris par des injections intrapéritoneales et intracéreébrales de patuline. *Mycopathologia et Mycologia applicata*, 54, 361–368.

CIEGLER, H., 1969. Tremorgenic toxin from *Penicillium palitans*. *Applied Microbiology*, 18, 128–129.

CIEGLER, A. & PITT, J. I., 1970. Survey of the genus *Penicillium* for tremorgenic toxin production. *Mycopathologia et Mycologia applicata*, 42, 119–124.

CLARDY, J., SPRINGER, J. P., BÜCHI, G., MATSUO, K. & WIGHTMAN, R., 1975. Tryptoquivaline and tryptoquivalone, two tremorgenic metabolites of *Aspergillus clavatus*. *Journal of the American Chemical Society*, 97, 663–665.

COLE, R. J., KIRKSEY, J. W. & WELLS, J. M., 1974. A new tremorgenic metabolite from *Penicillium paxilli*. *Canadian Journal of Microbiology*, 20, 1 159–1 162.

COLE, R. J., KIRKSEY, J. W., MOORE, J. H., BLAKENSHIP, B. R., DIENER, U. L. & DAVIS, N. D., 1972. Tremorgenic toxin from *Penicillium verruculosum*. *Applied Microbiology*, 24, 248–256.

DIENER, U. L., DAVIS, N. D., MORGAN-JONES, G., CUNNINGHAM, J. P., LANGFORD, J. W. & JOHNSON, W. C., 1975. Bermuda grass tremors in cattle in Alabama. *Highlights*, 22, 12.

FAYOS, J., LOKENSGARD, D., CLARDY, J., COLE, R. J. & KIRKSEY, J. W., 1974. Structure of verruculogen, a tremor producing peroxide from *Penicillium verruculosum*. *Journal of the American Chemical Society*, 96, 6 785–6 787.

GLINSUKON, T., YUAN, S. S., WIGHTMAN, R., KITAURA, Y., BÜCHI, G., SHANK, R. C., WOGAN, G. N. & CHRISTENSEN, C. M., 1974. Isolation and purification of cytochalasin E and two tremorgens from *Aspergillus clavatus*. *Plant Foods for Man*, 1, 113–119.

HOU, C. T., CIEGLER, A. & HESSELTINE, C. W., 1970. Tremorgenic toxins from *Penicillia*. II. A new tremorgenic toxin, tremortin B, from *Penicillium palitans*. *Canadian Journal of Microbiology*, 17, 599–603.

HOU, C. T., CIEGLER, A. & HESSELTINE, C. W., 1971. Tremorgenic toxins from *Penicillia*. III. Tremortin production by *Penicillium* species on various agricultural commodities. *Applied Microbiology*, 21, 1 101–1 103.

JACQUET, J., BOUTIBONNES, P. & CICILE, J. P., 1963. Observations sur la toxicité d' *Aspergillus clavatus* pour les animaux. *Bulletin de l'Académie Vétérinaire de France*, 36, 199–207.

MOREAU, C. & MOREAU, M., 1960. Un danger pour le bétail nourri de plantules fourragères cultivées en germoir; la pollution d' une moisissure toxique, l' *Aspergillus clavatus*, cause des accidents mortels. *Compte rendu de l'Académie d' Agriculture de France*, 46, 441–445.

PORTER, J. K., BACON, C. W. & ROBBINS, J. D., 1974. Major albaloids of a *Claviceps* isolated from toxic Bermuda grass. *Agricultural and Food Chemistry*, 22, 838–841.

SUZUKI, T., TAKEDA, M. & TANABE, H., 1971. A new mycotoxin produced by *Aspergillus clavatus*. *Chemical and Pharmaceutical Bulletin*, 19, 1 786–1 788.

WILSON, B. J. & WILSON, C. H., 1964. Toxin from *Aspergillus flavus:* Production on food materials of a substance causing tremors in mice. *Science*, 144, 177–178.

WILSON, B. J., WILSON, C. H. & HAYES, W. A., 1968. Tremorgenic toxin from *Penicillium cyclopium* grown on food materials. *Nature, London*, 220, 77–78.

YAMAZAKI, M., SUZUKI, S. & MIYAKI, K., 1971. Tremorgenic toxins from *Aspergillus fumigatus* Fres. *Chemical and Pharmaceutical Bulletin*, 19, 1 739–1 740.

Printed by and obtainable from The Government Printer, Private Bag X85, Pretoria, 0001