Tremorgenic neuromycotoxicosis in 2 dogs ascribed to the ingestion of penitrem A and possibly roquefortine in rice contaminated with Penicillium crustosum

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ABSTRACT
Two dogs developed alarming tremorgenic nervous stimulation shortly after ingesting discarded rice that had been forgotten in a refrigerator for an undetermined period and that was covered with a grey-green mould. Both dogs exhibited vomition followed by slight salivation, tremors and ataxia and 1 showed such severe agitation and seizures that it necessitated anaesthesia with thiopentone followed, on recovery, by xylazine. The other dog was only sedated with xylazine. They made an uneventful recovery. The rice vomitus yielded a pure culture of Penicillium crustosum. On chemical analysis it was negative for organochlorine, organophosphor and carbamate insecticides, as well as for strychnine, but contained 2.6 µg/g of the mycotoxins penitrem A as well as 34 µg/g of roquefortine as determined by LC-MS and confirmed by MS-MS. This is the 1st South African case of naturally occurring penitrem A intoxication as well as the 1st case where quantification of the levels of mycotoxins in dog vomitus is reported. The tremorgenicity of roquefortine and its contribution towards this syndrome, is questioned.

Key words: dogs, mouldy rice, neuromycotoxicosis, Penicillium crustosum, penitrem A, roquefortine, tremorgen.

INTRODUCTION
Penicillium crustosum Thom occurs in the air and soil and on various substrates such as food, meat products and feed. In addition is known as a weak pathogen of pomaceous and citrus fruit as well as a mycotoxigenic species that may produce several secondary metabolites that act as mycotoxins, including the tremorgenic penitrem A–F as well as the mildly neurotoxic roquefortine.

The utilisation of different Penicillium spp. (e.g. P. roqueforti and P. camemberti) for the production of specific types of cheese is well known. The fact that certain of these fungi may produce toxic metabolites such as the alkaloid roquefortine and PR toxin is, however, not common knowledge.

In man, eating of grossly overripe or mouldy cheese or other severely mouldy food hardly ever occurs because of its offensive smell, taste and nature. Dogs are, however, not as discriminating and toxicity due to ingestion of such products is on record.

Penitrem A (Fig. 1) is one of a group of 6 related penitremes and is closely-related to the tremorgenic paspalines produced by Claviceps paspali on Paspalum spp., the cause of paspalum staggers. This tremorgenic mycotoxin has a single nitrogen atom in a substituted indole moiety and a multi-ring structure derived from mevalonate units. It is produced by various Penicillium spp. but most commonly by P. crustosum isolates. By common agreement the trivial name tremortin A earlier in use for the toxin, was replaced with penitrem A.

Catović et al. regarded the mechanism of action to be in some way similar to that of strychnine in that it inhibits the inhibitory neurotransmitter glycine in the central nervous system. In particular the glycine concentration in the brain is lowered and the tremors can be abolished by mephenesin or nalorphine that raises the glycine level. The tremors are thus most probably of supraspinal origin. Later Norris et al. concluded that the mycotoxin acts by interfering with the glutamate, aspartate and GABA amino acid neurotransmitter release mechanisms. This is expected to result in anomalus release of both inhibitory and excitatory transmitters at central and peripheral synapses and the loss of the neural coordination controlling muscle action.

The i.p. LD₅₀ of penitrem A for mice is 1.05 mg/kg whereas dogs died at i.p. doses of 0.5 mg/kg and higher. In addition to the above 2 species, rats, rabbits, guinea-pigs, hamsters, chickens, calves, sheep and swine are susceptible to this neurotoxic substance. The signs of intoxication are similar in all species and set in within 5–10 min after i.p. or p.o. administration. Sustained tremors, ataxia and muscular rigidity progress in a dose-related fashion to fatal clonic or tetanic convulsions. Agitation, hyper-excitability and a change in temperament may be seen. In dogs, vomition often occurs and may be life-saving. An unusual feature of this neurotoxin is that it is also hepatotoxic resulting in dose-related centrilobular hepatic haemorrhage and necrosis in dogs and fatty changes in calves with concurrent elevation of serum aspartate transaminase. Owing to the tremors a sharp rise in creatine kinase is usually a feature of this neurotoxin is that it is also hepatotoxic resulting in dose-related centrilobular hepatic haemorrhage and necrosis in dogs and fatty changes in calves with concurrent elevation of serum aspartate transaminase. Owing to the tremors a sharp rise in creatine kinase is usually a feature of this neurotoxin. Recovery from intoxication is usually complete and without sequelae. Poisoning in dogs due to ingestion of P. crustosum-contaminated cream cheese, mouldy walnuts and a severely mouldy hamburger bun has been ascribed to penitrem A. The signs of intoxication were indistinguishable from those described with the purified toxin. No data could, however, be traced on the oral toxic dose of penitrem A to the dog.
Roquefortine (a diketopiperazine) is a 5-nitrogen-containing molecule (Fig. 2). It is a stable metabolite produced by *P. roqueforti*, *P. crustosum* and *P. chrysogenum*\(^{11}\). It has been isolated from processed cheese\(^{15}\) and has even been implicated in human intoxication from mouldy beer invaded by *P. crustosum*.

The i.p. LD\(_0\) first reported in mice was 15–20 mg/kg with opisthotonoid seizures elicited by external stimuli during prostration and atonic posture (Fraysinnet et al. cited in Scott et al.\(^{15}\)). This could, however, not be confirmed by Arnold et al.\(^{15}\) who found the LD\(_0\) ranging from 169–189 mg/kg in male and female Swiss-Webster and C17 mice. The signs of intoxication were hypokinesia and standing on their hind legs for prolonged periods of time and eventually, in those that were dying, quiescence, adipsia and anorexia No tremorgenic signs were reported. Roquefortine and penitrem A were isolated from cultures of *Penicillium commune* obtained from mouldy cottonseed meal during biological trials using neurotoxicity in day-old cockerels to monitor the isolation\(^{15}\). No dosages are given but the fractions containing the 2 individual neurotoxins were differentiated by the roquefortine resulting in paralysed while the penitrem A resulted in paralysis when, at our request, it was transferred to the owner’s home to sample for fungal spores. Fungal colonies which developed on incubation at 25 °C were identified. Mycologia of the kitchen yielded only the usual *Cladosporium* spp. dominated range of fungi one would expect.

**Fig. 1: Structural formula of penitrem A.**

**Fig. 2: Structural formula of roquefortine.** Note (arrow) the isoprene unit (in a novel arrangement as a 1,1-dimethylallyl group) on position 3 of the indole nucleus.

**RESULTS**

**Mycological investigation**

A pure culture of a fungus was isolated from the vomitus, then inoculated and grown according to the regime of Pitt\(^{6}\). This briefly consists of point-inoculating 3 Petri dishes of Czapek yeast autolysate agar (CYA)\(^{22}\) and 1 Petri dish each of malt extract agar (MEA)\(^{32}\) and 25 % glycerol nitrate agar (G25N)\(^{5}\). The MEA, G25N and 1 CYA plate were incubated at 25 °C, and the 2 remaining CYA plates at 37 °C and 5 °C, respectively. Macroscopic and microscopic morphology on all media was examined after 7 days’ incubation. The isolate was identified as *P. crustosum* Thom, a terverticilate *Penicillium* in the subgenus *Penicillium*, series *Viridicata* Raper & Thom ex Pitt\(^{25,37,42}\).

Petri dishes with potato carrot agar and 1.5 % malt extract agar, respectively, were exposed to the air for 20 minutes in the kitchen/scullery and the 2 refrigerators at the owner’s home to sample for fungal spores. Fungal colonies which developed on incubation at 25 °C were identified. Mycologia of the kitchen yielded only the usual *Cladosporium* spp. dominated range of fungi one would expect.

**Chemical investigations**

**Analysis for mycotoxins**

**Sample treatment.** Vomitus (10 g wet mass) was introduced into a 500-ml stopped flask and shaken up twice with dichloromethane:methanol:ethyl acetate (2:1:3, v/v/v) containing 0.1 % formic acid (100 ml, followed by 50 ml). The extracts were filtered (Whatman No. 1), combined, and evaporated to dryness under reduced pressure at 50 °C on a rotatory evaporator. The residue was taken up in methanol (5 ml) and a portion of the methanol solution (0.5 ml) was run onto a Bond Elute C18 cartridge (500 ml) (Varian, USA) which was then successively eluted with 1 ml methanol:water (1:1, v/v) and 2 ml methanol. Prior to LC-MS analysis, the methanol:water and methanol fractions were filtered using a Spin-X (Corning, New York) centrifugal filter.

**LC-MS and LC-MS-MS analyses.** Chromatography was performed on a Waters Symmetry C18 column (5 μm, 4.6 x 150 mm) (Milford, USA), using a TSP P4000 pump and an AS3000 autosampler (San Jose, USA). A gradient mobile phase consisting of mixtures of methanol and 0.1 % HCOOH and 0.01 M ammonium acetate was used. The methanol level of the mobile phase, starting at 25 % (2 min initial hold) was raised to 60 % over 15 min, then to 95 % over 5 min, and held for 10 min. The flow rate was 0.7 ml/min.
and 20 µL of the filtered methanol-water, or methanol fractions were injected.

The HPLC system was coupled to a Finnigan MAT, LCQ ion trap mass spectrometer operated with an atmospheric pressure chemical ionisation (APCI) interface (San Jose, USA). Spectral data were acquired in both MS in full scan and MS-MS in full scan positive ion modes. The MS ion injection time was set to 400 ms with a total of 2 micro-scans per second. A vaporisation temperature at 350 °C, a sheath gas rate at 25 units nitrogen, a corona discharge voltage of 4.5 V and a capillary temperature of 200 °C were used.

Results

LC-MS analyses, performed in full scan mode, demonstrated the presence of roquefortine C (m/z 390, MH⁺) in both the methanol–water and the methanol fraction. Penitrem A (m/z 634, MH⁺) was only detected in the methanol fraction. These mycotoxins were initially identified on the basis of their MH⁺ ions and retention time comparison with authentic specimens (Fig. 3a, peaks at 14.5 and at 25.3 min). Protonated molecular ions corresponding to penitrems B, C, D, E or F could not be detected in either of the fractions of the sample.

Acquisition of mass spectral data in MS-MS mode greatly increased the selectivity and signal to noise ratio (and hence the detection limits) of the analytical procedure (see Fig. 3b). Ions at m/z 390 and 634, corresponding to the MH⁺ ions of roquefortine C and penitrem A, respectively, were fragmented and analysed in the MS-MS stage. Under the MS-MS conditions applied in this investigation, the m/z 390 (MH⁺) ion of roquefortine fragmented to afford m/z 322 (loss of imidazole C₃H₄N₂), and 193 ions (Fig. 4a), whereas the m/z 634 (MH⁺) ion of penitrem A fragmented to afford m/z 616 and 588 ions (Fig. 4b), corresponding to the consequential loss of water and the elements of acetone, respectively. The loss of a mole of acetone is of particular diagnostic significance in that it can be attributed to the loss of the bridging –C(CH₃)₂–O– ether linkage present in penitremin type mycotoxins produced by Penicillium species. The MS-MS spectra of the m/z 390 and 634 ions, shown in Fig 4a,b, corresponds with those of authentic specimens of roquefortine C and penitrem A, respectively.

Quantification, performed in MS-MS mode, demonstrated the presence in the vomitus of 34 µg/g wet mass of roquefortine C (sum of methanol and methanol-water fractions contributions) and 2.6 µg/g wet mass of penitrem A.

Analysis for other possible neurotoxic substances

Analysis of the remainder of the vomitus (11 g) for organochlorine, organophosphor and carbamate insecticides by GC/MS at the Veterinary Institute, Oslo, and (7 g) for strychnine by TLC at the Division of Toxicology at the Onderstepoort Veterinary Institute, Pretoria, proved to be negative.

DISCUSSION

In South Africa the usual toxicological differential diagnoses in dogs considered and tested for when agitation, trembling and nervous stimulation eventually progressing to convulsive seizures, prostration and death are encountered, are (in sequence of importance) poisoning by pesticides of the organochlorine, organophosphor or carbamate groups and secondly, strychnine. Lately fluoroacetate (illegally obtained and used) has been added as poisoning does occur and a practicable test for it has been developed. Lead poisoning is rare but is occasionally encountered. Metaldehyde poisoning, even more rarely encountered here, must also be considered (TW Naudé, pers. obs., 2001).

The oral toxic dose of penitrem A for the dog is unknown (vide supra). Unfortunately it was also impossible to arrive at a reasonable estimate of what dose had resulted in intoxication in this particular instance. Only the amount of penitrem A in the a specimen of the 1st dog’s vomitus, which had been kept in the owner’s refrigerator for a week before it was frozen and finally analysed, is known. Other unknown factors are the amounts...
of infected rice actually eaten by each dog and disgorged later. Finally the amount of toxin that could have been produced during the c. 1-week storage of the vomitus in the same fridge where the mycotoxins had initially been produced on rice, has to be considered. Nevertheless in our opinion a diagnosis of penitrem A poisoning (possibly influenced by the presence of roquefortine) is justified. Consequently, this should now be added to the list of possible neurotoxicities of dogs in South Africa. Fortunately, suitable qualitative and semi-quantitative analytical TLC techniques for penitrem A 20 and roquefortine 28 are available and could be used routinely in a diagnostic facility.

The air samples from the refrigerators as well as the kitchen were free of P. crustosum. Although the owner of the dogs stated that they had earlier kept some pears in the kitchen and the fungus is known to be a weak pathogen of, amongst others, pomaceous fruit 29,30 this could not be related to the origin of the contamination.

The refrigerator in which the incriminated rice infection had developed was at a maximum temperature of 3 °C and a minimum of 1 °C. One of the diagnostic characteristics within the subgenus Penicillium is the ability of these species to germinate and grow at low temperatures 22,24 and produce toxins 27. It was demonstrated that P. crustosum grown at 4 °C on rice produced considerable amounts of penitrem A, which peaked at 50–85 days with a high level still being present at 120 days. By contrast, at 20 °C, peak production of toxin on this substrate was at 10–25 days and by 50 days the level had been depleted greatly 31. This particular isolate also proved to produce penitrem A when grown on rice at 10 °C (T Rundberget, Veterinary Institute, Oslo, pers. comm., 2001).

Penitrem A intoxication in dogs has clearly been established by administration of purified toxin 26 and the signs of intoxication are indistinguishable from those in field cases where it occurs concurrently with roquefortine. In the 3 natural outbreaks of intoxication ascribed to penitrem A poisoning 24,25,28 the presence of roquefortine was not excluded. The practical significance of roquefortine, its role in intoxication of dogs in particular and, indeed, of whether it is a tremorgen or not, arises. Although it is widely grouped with the other tremogens in general there appears to be only the cited reference of Fraysinet and Fraysinet in Scott et al. 23 where convulsive seizures in mice at 15–20 mg/kg is reported. This finding was, however, in contrast to later work where hypokinesia and quiescence occurred at a very much higher LD 3. In fact Wagener et al. 2, using day-old chicks to monitor their isola-
tion, clearly differentiated penitrem A from roquefortine on grounds of the tremorgenic activity of the former in con-
trast to paralytic action of the latter. Yamazaki (1980) 28 groups roquefortine chemically with fumitremorgen and verruculogen but points out that it differs structurally from these 2 by having an isoprene unit (in a novel arrangement as a 1,1-dimethylallyl group) on position 3 of the indole nucleus in stead of the usual 2 position (see arrow in Fig. 2).

In Canada, roquefortine was present in the stomach contents of a significant number of dogs that had died of or had exhibited signs resembling strychnine poisoning but where strychnine tests were negative. The toxicity was ascribed to roquefortine although quantification was not done and it was admitted that no reports on the clinical signs of this intoxication in the dog were available 28,31. Although penitrem A is mentioned in these publications, it was not excluded in either case. Braselton and Rumler 2 reported the presence of roquefortine in the stomach content of 7 dogs that had been tested for strychnine with negative results. However, significantly, in 6 of these cases where sufficient sample were available, penitrem A was also present and they were the first to speculate on the possible synergistic action of these 2 toxins. Quantification was, however, unfortunately again not reported. In a further 34 out of 37 specimens ranging from stomach contents and vomitus to mouldy food, the presence of both toxins were confirmed by MS-MS and in the other 3 the suspected presence of peni-
trem A was too low to confirm by MS-MS (E Braselton, Michigan State University, pers. comm., 2000).

There appear to be little justification in diagnosing roquefortine poisoning in dogs. There are no data on its toxicity in the dog, no definite data confirming its tremorgenic potential (in fact it rather appears to have a paralytic action) and it has a relatively low toxicity in mice. It would appear that the chemically con-

firmed presence of roquefortine in the vomitus in our case (and indeed the gastric contents of the Canadian dogs) may perhaps have been just incidental and only an indication that food contami-
nated with a roquefortine-producing Penicillium spp. had been ingested.

Treatment of this intoxication should be as for strychnine, namely induction of vomition (if the dog is not in a convulsive state or if this is not imminent) followed by anaesthesia with pentobarbitone. If on recovery from the latter more sedation is required, xylazine (which was used with success) seems indicated. It is of interest to note that Lowes et al. 19 found the tremor-
genic state they had ascribed to roque-
fortine poisoning totally unresponsive to diazepam. Peterson et al. 26 also found that diazepam to some extent controlled the clinical signs of the tremorgen verru-
culogen but did not abolish it as com-
pared to the barbiturates. It seems probable that this could be the same with penitrem A. The use of activated charcoal per os, as in most poisonings, is clearly indicated. In view of the fact that liver damage was reported in dogs with penitrem A intoxication 25, liver support-
ive therapy should also be considered.

The scavenging nature of dogs and their interest in and occasional predilection for garbage and foul smelling food probably make this the species most prone to penitrem A poisoning, particularly as P. crustosum is an ubiquitous spoilage organism of food 28,29,34,35. The reason why this intoxication has not been diagnosed previously in South Africa is probably that veterinarians had been unaware of this neuromyotoxicosis and had not considered it.

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