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 toxicosis and also 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.

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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 cucurbits[29], but it is also known as an ubiquitous spoilage organism that may cause post-harvest rot of fruit. It also commonly contaminates foodstuffs which may then become toxic[16,28,32]. Strains within this species are known to produce several secondary metabolites that act as mycotoxins, including the tremorgens penitrem A–F as well as the mildly neurotoxic roquefortine[11,17,21].

The utilisation of different *Penicillium* spp. (*e.g. P. roqueforti* and *P. camembertii*) 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[12] 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[19,31,32].

Penitrem A (Fig. 1) is one of a group of 6 related penitrems and is closely-related to the tremorgenic paspalines produced by *Claviceps paspali* on *Paspalum* spp., the cause of paspalum staggers[20]. 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[3]. By common agreement the trivial name tremortin A earlier in use for the toxin, was replaced with penitrem A[28].

Čatovič *et al.*[6] 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.*[24] concluded that the mycotoxin acts by interfering with the glutamate, aspartate and GABA amino acid neurotransmitter release mechanisms. This is expected to result in anomalous 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. LD_{50} of penitrem A for mice is 1.05 mg/kg[15] whereas dogs died at i.p. doses of 0.5 mg/kg and higher[13]. In addition to the above 2 species, rats, rabbits, guinea-pigs, hamsters, chickens, calves, sheep and swine are susceptible to this neurotoxic substance[9,10]. 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, hyperexcitability 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 activity. Owing to the tremors a sharp rise in creatine kinase activity also occurs. Recovery from intoxication is usually complete and without sequelae. Poisoning in dogs due to ingestion of *P. crustosum*-contaminated cream cheese[2], mouldy walnuts[33] and a severely mouldy hamburger bun[4] has been ascribed to penitrem A. The signs of intoxication were indistinguishable from those described with the purified toxin[13]. No data could, however, be traced on the oral toxic dose of penitrem A to the dog.

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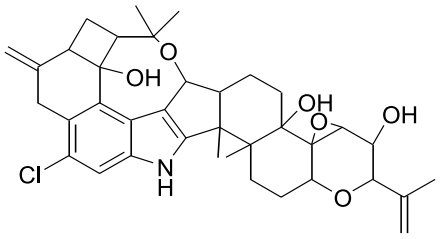

Fig. 1: **Structural formula of penitrem A.**

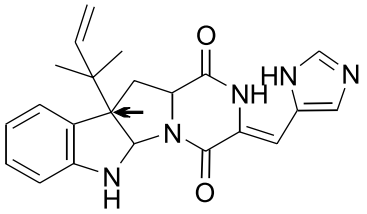

Fig. 2: **Structural formula of roquefortine. Note** (arrow) **the isoprene unit** (in a novel arrangement as a 1,1-dimethylallyl group) **on position 3 of the indole nucleus.**

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*[30]. It has been isolated from processed cheese[35] and has even been implicated in human intoxication from mouldy beer invaded by *P. crustosum*[7].

The i.p. LD_{50} first reported in mice was 15–20 mg/kg with opisthotonoid seizures elicited by external stimuli during prostration and atonic posture (Fraysinnet and Frayssinet cited in Scott *et al.*[36]). This could, however, not be confirmed by Arnold *et al.*[1] who found the LD_{50} 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[38]. No dosages are given but the fractions containing the 2 individual neurotoxins were differentiated by the roquefortine resulting in paralysis while the penitrem A resulted in tremors. Although roquefortine has been implicated in severe excitatory neurotoxicity of dogs, resembling strychnine poisoning[18,19,31], no experimental data on its toxicity in dogs could be traced.

Roquefortine and penitrem A are produced concurrently by *Penicillium crustosum*[21] but no studies have been done on the effects of the concurrent exposure to and possible synergistic effects of the 2 toxins[5].

Although penitrem A/roquefortine toxicity in dogs has been recorded as early as1979[2] and 1988[31], respectively, this is the 1st report of this intoxication in South Africa.

CASE REPORT

One evening a dog owner discarded the contents of a plastic-covered bowl of cooked rice which had been forgotten at the bottom in his refrigerator for an unknown period and which had become overgrown with a dark-green mould layer, on his compost heap in the garden. His 2 dogs discovered it and ate it within an hour. About 2 h later his 2-year-old, spayed Miniature Schnauzer bitch (mass *c.* 20 kg) startedshivering violently and vomited repeatedly, disgorging a rice-like substance. He took her to his veterinarian where the dog presented with ataxia, muscle tremors, polypnoea, severe agitation and generalised seizures. The dog was anaesthetised with thiopentone (Intraval, Rhône-Poulenc) i.v. (25 mg/kg), treated s.c. with atropine sulphate (Centaur Labs) (0.1 mg/kg) and clanobutin (Bykahepar, Centaur Labs) (27 mg/kg) and hospitalised. Two hours later it was sitting up in its cage but was still exhibiting muscle tremors and slight ataxia. Xylazine (Rompun, Bayer) (10 mg/kg) was given i.m. to sedate the dog overnight.

On returning home *c.* 1 hour later the owner's 2nd dog, a 3-year-old cross-bred Schnauzer (mass *c.* 25 kg), was also shivering and exhibiting loss of balance. He gave the dog some home medication by mouth which induced vomition. This dog was then, 2 hours after the 1st one, also taken to the consulting room and presented with slight salivation and muscle tremors. It was similarly treated with atropine, clanobutin and xylazine and also hospitalised.

Both dogs were clinically normal the next morning and were discharged.

The owner's son fortunately collected vomitus from the 1st dog off the floor and kept it in their refrigerator in a tightly closed wide-mouth jar for *c.* 1 week when, at our request, it was transferred to the veterinary consulting room where it was kept frozen. At this stage a clearly visible green fungal covering was present on the outer surface of the rice grains in the bottle. The vomitus was kept in a frozen state up to the stage that the mycological investigation and, as far as practicable also the chemical analyses, were carried out.

RESULTS

Mycological investigation

A pure culture of a fungus was isolated from the vomitus, then inoculated and grown according to the regime of Pitt[28]. This briefly consists of point-inoculating 3 Petri dishes of Czapek yeast autolysate agar (CYA)[27] and 1 Petri dish each of malt extract agar (MEA)[4,28] and 25 % glycerol nitrate agar (G25N)[27]. 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 microscopical 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[28,37,40].

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. Mycoflora 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 stoppered 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 mg) (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 × 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

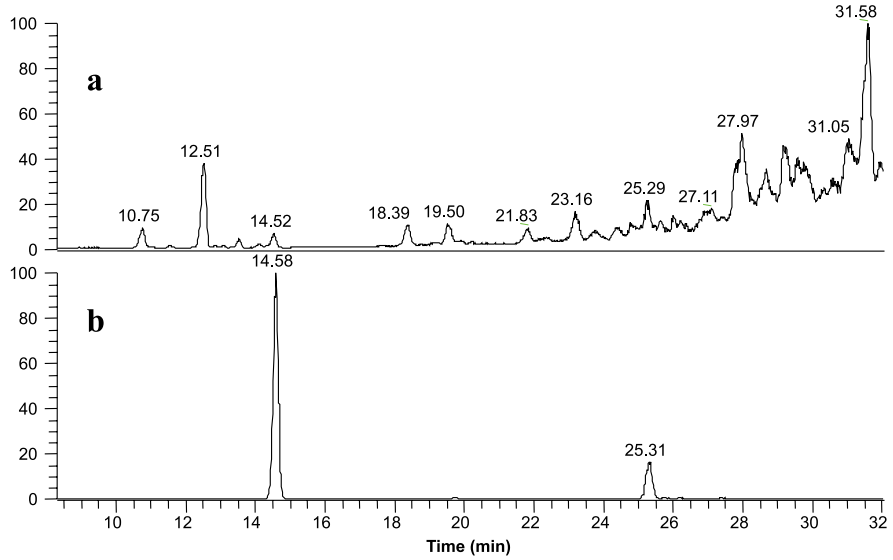

Fig. 3: (a) **LC-MS and** (b) **LC-MS-MS chromatograms of the methanol fraction of the vomitus extract.**

and 20 $\mu\ell$ 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 $C_3H_4N_2$), and 193 ions (Fig. 4a), whereas the m/z 634 (MH^+) ion of penitrem A fragmented to afford m/z 616 and 558 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 $-C(CH_3)_2-O-$ ether linkage present in penitrem 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.

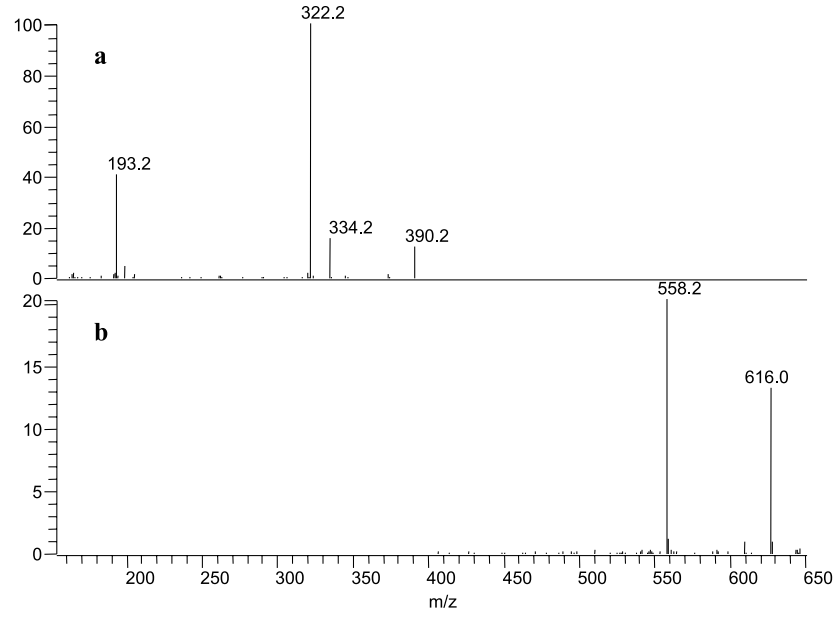

Fig. 4: **MS-MS spectra of** (a) **roquefortine C and** (b) **penitrem A.**

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[22]. Lead poisoning is rare but is occasionally encountered. Metaldehyde poisoning, even more rarely encountered here, must also be considered (T W 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[34] 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 had stated that they had earlier kept some pears in the kitchen and the fungus is known to be weak pathogen of, amongst others, pomaceous fruit[29] , 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[23,28] and produce toxins[28]. 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[16]. 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[13] 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[2,14,33] 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 tremorgens in general there appears to be only the cited reference of Fraysinet and Fraysinet in Scott *et al.*[36] 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[1]. In fact Wagener *et al.*[38], using day-old cockerels to monitor their isolation, clearly differentiated penitrem A from roquefortine on grounds of the tremorgenic activity of the former in contrast to paralytic action of the latter. Yamazaki (1980)[41] 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[19,31]. Although penitrem A is mentioned in these publications, it was not excluded in either case. Braselton and Rumler[5] 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 penitrem 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 confirmed 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 contaminated 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 tremorgenic state they had ascribed to roquefortine poisoning totally unresponsive to diazepam. Peterson *et al.*[26] also found that diazepam to some extent controlled the clinical signs of the tremorgen verruculogen but did not abolish it as compared 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[13], liver supportive therapy should also be considered.

The scavenging nature of dogs and their interest in and occasional predilection for garbage and foul smelling food[8] probably make this the species most prone to penitrem A poisoning, particularly as *P. crustosum* is an ubiquitous spoilage organism of food[25,29,39,40]. The reason why this intoxication has not been diagnosed previously in South Africa is probably that veterinarians had been unaware of this neuromycotoxicosis and had not considered it.

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