Putative *Aspergillus niger*-induced oxalate nephrosis in sheep

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ABSTRACT

A sheep farmer provided a maize-based brewer's grain (*mieliemaroek*) and bales of *Eragrostis curvula* hay to ewes and their lambs, kept on zero-grazing in pens. The 'mieliemaroek' was visibly mouldy. After 14 days in the feedlot, clinical signs, including generalised weakness, ataxia of the hind limbs, tremors and recumbency, were noticed. Six ewes died within a period of 7 days. A *post mortem* examination was performed on 1 ewe. The carcass appeared to be cachectic with mild effusions into the body cavities; mild lung congestion and pallor of the kidneys were observed. Microscopical evaluation revealed nephrosis and birefringent oxalate crystals in the renal tubules when viewed under polarised light. A provisional diagnosis of oxalate nephrosis with subsequent kidney failure was made. Amongst other fungi, *Aspergillus niger* was isolated from 'mieliemaroek' samples submitted for fungal culture and identification. As *A. niger* is known to synthesise oxalates, a qualitative screen to detect oxalic acid in the *mieliemaroek* and purified *A. niger* isolates was performed using high-performance liquid chromatography (HPLC). Oxalic acid was detected, which supported a diagnosis of soluble oxalate-induced nephropathy.

Key words: *Aspergillus niger,* kidney, nephrosis, oxalates, poisoning, sheep.

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INTRODUCTION

In South Africa soluble oxalate poisoning is predominantly a ruminant (especially sheep) problem[5]. It is mainly caused by oxalate-containing plants that are particularly palatable for livestock[4,5]. In acute poisoning, excessive soluble oxalates released in the rumen will be absorbed into the bloodstream and bind with calcium and/or magnesium. Hypocalcaemia occurs when unadapted animals suddenly ingest a relatively large amount of oxalate-containing plants or even when adapted animals consume excessive amounts (for instance during droughts) and the capacity of the rumen to detoxify all the ingested oxalates is exceeded[4,5].

On the other hand, subacute poisoning follows damage to and oxalate crystallisation in especially the kidney tubules, causing nephrosis and uraemia, characterised by increased blood urea nitrogen (BUN) and creatinine concentrations. *Post mortem* changes include, amongst others, ascites, hydrothorax, perirenal and subcutaneous oedema, haemorrhages in different organs and pale, oedematous and swollen kidneys. Microscopically the typical oxalate crystals, seen under polarised light, in the kidney tubules are of diagnostic value[5,10].

Worldwide a large number of plants may contain soluble oxalates in hazardous concentrations. In South Africa oxalate-containing plants such as *Oxalis* and *Rumex* species are often incriminated[5,10]. Besides phytogenous causes, ethylene glycol[3], vitamin C (ascorbic acid)[1] and *Aspergillus niger*[2,14] are also implicated as a cause of oxalate nephrotoxicoses in ruminants.

Although *Aspergillus niger*-induced oxalate nephrosis is often listed in bibliographies, there is a dearth of information available in the scientific literature reporting outbreaks.

CASE HISTORY

A farmer in the Warden district of the eastern Free State kept 200 South African Mutton Merino ewes with their 2–3-month-old suckling lambs in a feedlot. There were 5 pens with 40 ewes and their lambs in each pen. A maize-based brewer's grain (*mieliemaroek*) was available in self-feeders *ad libitum* and bales of *Eragrostis curvula* hay were supplied. The sheep had no access to any other feed or grazing.

The animals were fed for approximately 14 days in the feedlot before clinical signs were noticed. The affected ewes were in a poor condition (condition score of 1.5–2), showed generalised weakness and nervous signs, such as ataxia of the hind limbs, body and head tremors, paresis and paralysis. Later the affected ewes became recumbent and died within 24 hours after the onset of clinical signs. Six ewes died within a period of 7 days.

A *post mortem* examination was performed on a ewe. Samples of the major organs were collected and fixed in 10 % buffered formalin, routinely processed and stained for microscopical evaluation. The *mieliemaroek* was noticeably mouldy and clumped together.

Clumps of the *mieliemaroek* were inspected; these felt warm when handled and on opening revealed black, gray, pink and white fungal growth (Fig. 1).

Representative samples of the maize-based brewer's grain was also collected and submitted for fungal culture and identification.

Fungal isolation and identification

The maize-based brewer's grain sample was directly plated to potato carrot agar (PCA) (20 g potato and 20 g carrot slices, boiled and extract used, 15 g agar) with 50 mg rifampicin (Sigma-Aldrich), using 6 replicate plates, and incubated for 4–7 days at 25 °C. Pure cultures were prepared from developing fungal colonies by transferring a small amount of spores and hyphae with a fine sterile needle with the aid of a stereomicroscope to new PCA plates.

Pure fungal isolates were incubated for at least 7 days at 25 °C before being identified based on morphological characteristics. All retrieved *Aspergillus* isolates were transferred to Czapek yeast extract agar, Czapek yeast extract agar with 20 % sucrose and malt extract agar as described by Klich and Pitt[6]. The inoculated plates were incubated at 5, 25 and 37 °C for 7 days, whereafter colony and microscopic characteristics were examined and recorded. Colony texture, colony reverse, mycelia, conidial heads, cleistothecia, sclerotia and soluble pigment when

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present, were determined under natural light at a window. The Methuen colour standard[7] was used to compare colony and exudate colour. The microscopic features were assessed at ×400 to ×1000 magnification using a light microscope and compared with species descriptions[6,11,13].

Oxalic acid analysis

A qualitative screen to verify the presence of oxalic acid in the maize-based brewer's grain sample and purified *A. niger* isolates was performed by high-performance liquid chromatography (Beckman, System Gold with Karat 32 software) according to the method as described by Libert[8] with some modifications. Briefly, the grain sample was divided into 2 samples and milled to obtain an even distribution and size of the grain particles. Five grams of each was weighed out and 25 mℓ 1 M hydrochloric acid (HCl) added. The samples were thoroughly mixed and placed in an oven for 15 min at 100 °C and then left at room temperature overnight. The next day the samples were filtered (Whatman number 1 filter) and centrifuged for 15 min at 2500 rpm. *Oxalis latifolia* (red garden sorrel), known to contain high concentrations of oxalic acid[5], was collected from the Poisonous Plant Garden, Faculty of Veterinary Science, Onderstepoort, and included in the analysis as a positive control. The plant material was dried overnight at 30 °C. Six grams of the dried plant material was chopped up, 50 mℓ 1 M HCl added, thoroughly mixed and placed in an oven for 15 min at 100 °C and then left at room temperature overnight. The following day the plant material was strained through gauze to remove most of the plant residue and then filtered (Whatman number 1 filter). Four selected isolates of *A. niger* were cultivated on potato dextrose agar (Biolab, Merck) for 7 days at 25 °C. The fungal growth of each isolate was removed from the culture plates with 10 mℓ 0.1 % orthophosphoric acid (H_3PO_4), homogenised (Diax 600, Heidolph) for 2 min and filtered (Whatman no. 1 filter).

A cartridge (Waters Sep-Pak cartridge for solid phase extraction; 200 mg, 4 mℓ) was prepared for each sample by regenerating it with 4 mℓ methanol (Merck) and then washed with 2 mℓ ultrapurified water (Milli-Q). The plant material and *mieliemaroek* samples were diluted 1:1 with ultrapurified water (Milli-Q) and 4 mℓ of the filtrates of each sample were placed onto the prepared cartridges. The first 2 mℓ of the eluent of each was discarded and the next 2 mℓ was collected and injected onto the HPLC column (RSpak KC-811 with a column size 8.0 mm ID by 300 mm length, Shodex, Japan). The mobile phase was 0.1 % H_3PO_4/H_2O with a flow rate of 0.8 mℓ/min, the injection volume of each sample was 25 µl and UV detection (Beckman System Gold 168 detector) at 220 nm. Glycolic acid and anhydrous oxalic acid (Fluka, Sigma-Aldrich) were used as standards and included when analysing the samples.

Fig. 1: **The mouldy maize-based brewer's grain** (*mieliemaroek*) **fed to the sheep.**

RESULTS

Macroscopic lesions

At necropsy the carcass appeared to be cachectic with mild hydrothorax and ascites. Slight lung congestion was present and the kidneys had a pale appearance.

Histopathology

Microscopical evaluation revealed nephrosis and oxalate crystals in the renal tubules. These crystals were birefringent under polarised light. A mild degeneration of the cerebellar granular layer as well as a few multifocal areas of cerebral vacuolisation were noticed and pulmonary congestion was evident.

A provisional diagnosis of oxalate nephrosis with subsequent kidney failure and a possible renal encephalopathy was made.

Fungal identification

Aspergillus flavus Link, *Aspergillus niger* Tiegh (Fig. 2), *Aspergillus terreus* Thom., *Paecilomyces* sp., *Penicillium* spp. and *Scopulariopsis* sp. were retrieved from the plated material.

HPLC analysis

Oxalic acid was detected in the *Oxalis* plant material, in the maize-based

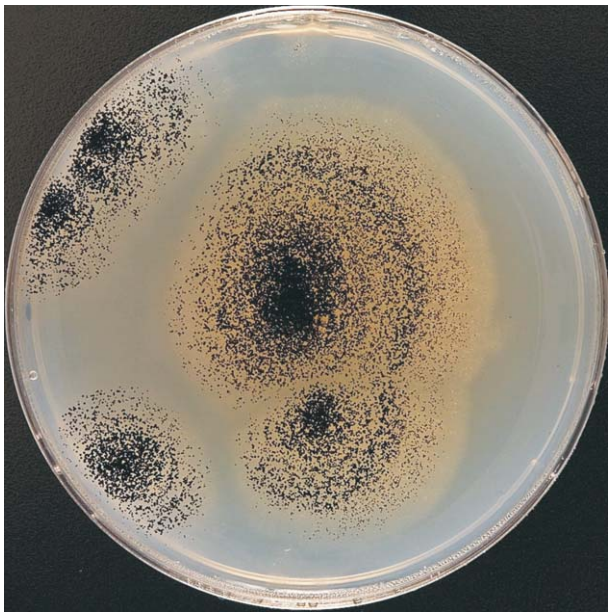

Fig. 2: ***Aspergillus niger*** **culture on potato dextrose agar.**

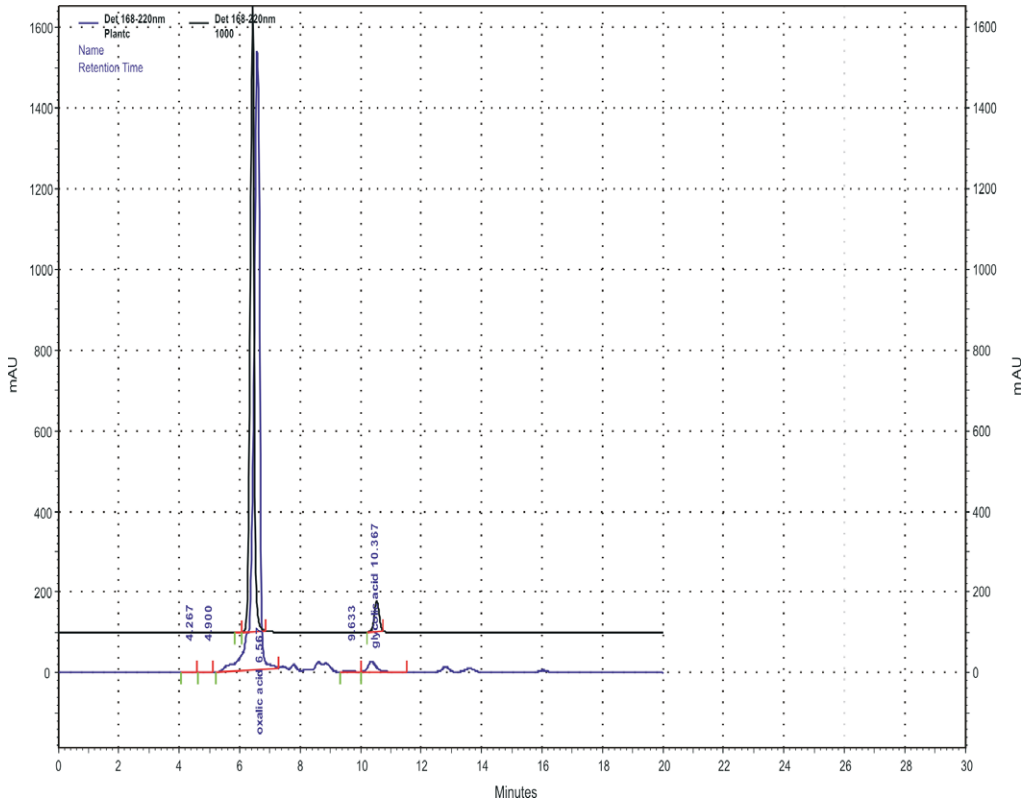

Fig. 3: **Chromatogram depicting peaks of oxalic acid** (standard; black) **and oxalic acid contained in *Oxalis latifolia* plant material** (blue)**.**

brewer's grain (*mieliemaroek*) sample as well as in the purified *A. niger* isolates (Figs 3–5).

DISCUSSION

The presence of oxalic acid in the *mieliemaroek* as well as the *A. niger* isolates supports a diagnosis of soluble oxalate-induced nephropathy. It is suspected that *A. niger* played an important role in the aetiology of oxalate nephrotoxicosis in this outbreak as the mouldy *mieliemaroek* made up a considerable proportion of the ration provided to the sheep. Although some of the other fungi isolated from the *mieliemaroek*, such as *Aspergillus flavus*[14] and *Penicillium* species[2,10,14], might also synthesise oxalates, *A. niger* is regarded as an important source[9,11,14]. Wilson and Wilson[14] reported that toxicity induced by *A. niger* strains in mice was primarily due

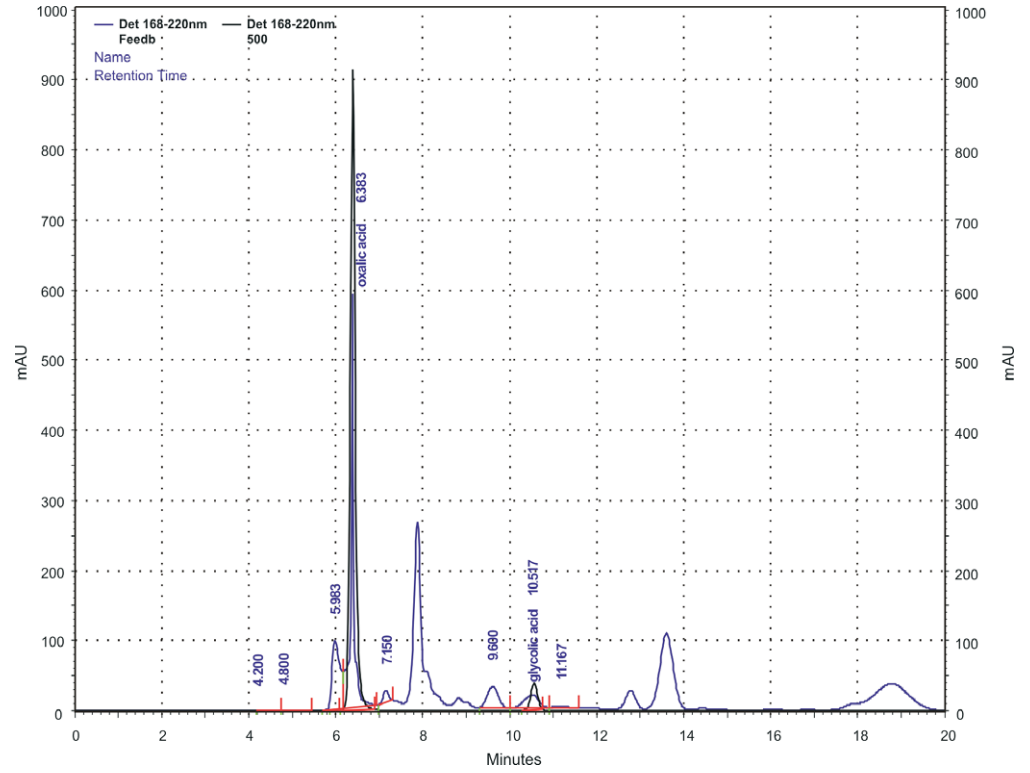

Fig. 4: **Chromatogram depicting peaks of oxalic acid** (standard; black) **and oxalic acid contained in the maize-based brewer's grain** (*mieliemaroek*) **sample** (blue).

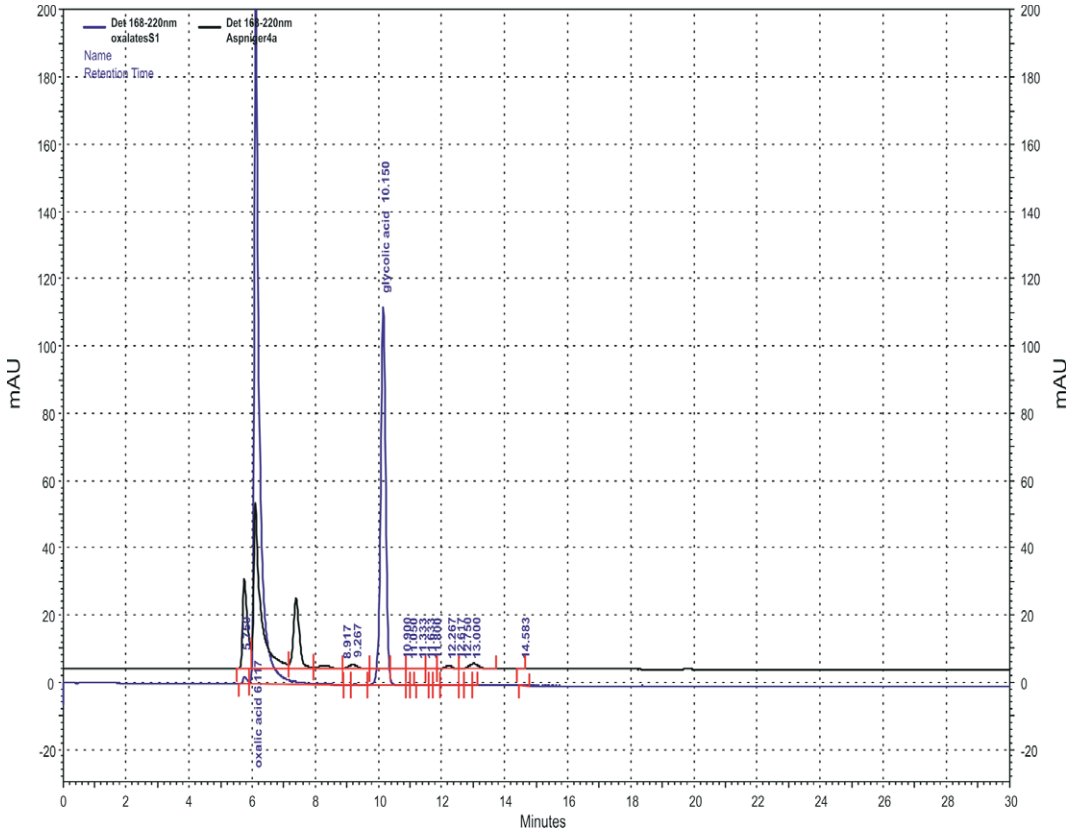

Fig. 5: **Chromatogram depicting peaks of oxalic acid** (standard; blue) **and oxalic acid contained in a purified *Aspergillus niger* isolate** (black)**.**

to the oxalate salts produced in large quantities under optimal culture conditions. The possibility of ingestion of other soluble oxalate-containing plants was considered, but this was not supported by the history and circumstances associated with the poisoning.

In addition to oxalates, strains of *A. niger* occurring on grapes have also been shown to produce ochratoxin A, a potent nephrotoxin[12]. However, ochratoxicosis is predominantly described in swine and poultry in veterinary medicine[2,5]. Another potential mycotoxin, isolated from *A. niger* is malformin A_1, but oral administration to male mice demonstrated very low acute toxicity[15].

Besides the histopathological evidence of cerebral and cerebellar lesions, attributed to renal encephalopathy, the anticipated hypocalcaemia and hypomagnesaemia[4,5,10] could also have contributed to the neurological manifestations. It is surmised that only the ewes were affected as the lambs were still nursing and probably ingested smaller quantities of the available feed. Furthermore, it is conceivable that the oxalic acid consumed will bind to calcium contained in the milk, forming poorly soluble chelates which will render the oxalates unavailable for absorption[5,10].

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