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Complex etiology and pathology of mycotoxic nephropathy in South African pigs

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Abstract Spontaneous nephropathy in pigs seen in South Africa was found to have multi-mycotoxic etiology involving several mycotoxins such as ochratoxin A (OTA), penicillic acid (PA) and fumonisin B_1 (FB_1) in addition to a not yet identified mycotoxin. Contamination levels of OTA were comparatively low (67–75 µg/kg) in contrast to high contamination levels of FB_1 (5,289–5,021 µg/kg) and PA (149–251 µg/kg). A heavy contamination with *Gibberella fujikuroi* var. *moniliformis* and *Penicillium aurantiogriseum* complex (mainly *P. polonicum*) was observed in the fed forages in contrast to the light contamination with *Aspergillus ochraceus, P. verrucosum* and *P. citrinum*. The pathomorphological picture of this nephropathy was found to differ from the classical description of mycotoxic porcine nephropathy as originally made in Scandinavia by the extensive vascular changes.

Keywords Ochratoxin A · Penicillic acid · Citrinin · Fumonisin B_1 · Mycotoxins · Mycotoxic nephropathy · Pigs

Abbreviations

AFs	Aflatoxins
BEN	Balkan endemic nephropathy
CIT	Citrinin
CA	Cyclopiazonic acid
DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
ERY	Erythroskyrin
FB_1	Fumonisin B1
FB_2	Fumonisin B2
GLI	Gliotoxin
ISL	Islanditoxin
KA	Kojic acid
LUT	Luteoskyirin
MPN	Mycotoxic porcine nephropathy
MCN	Mycotoxic chicken nephropathy
MON	Moniliformin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OTA	Ochratoxin A
OTC	Ochratoxin C
OOS	Oosporein (isoosporin)
PenA	Penitrem A
PAT	Patulin
PA	Penicillic acid
ppb	µg/kg
T-2	T-2 toxin

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UM Unknown metabolite
XA Xanthomegnin
ZEA Zearalenone.

Introduction

The mycotoxic nephropathy (MN) is a renal disorder caused by alimentary ingestion of secondary fungal metabolites possessing nephrotoxic properties, which contaminate feeds and foods made mainly from cereals or fibrous plants, and kept in storehouse conditions with increased humidity. Since its discovery, the disease has been named in different ways: nephrosis provoked by moulds, chronic interstitial fibrosis of kidneys, chronic interstitial nephritis, ochratoxicosis, mycotoxic nephropathy, etc. Some varieties were found with the manifestation of the disease, especially with the clinicomorphological picture, which in many cases is influenced by the combined nephrotoxic effect of several nephrotoxic mycotoxins (Stoev et al. 1998a, 2001, 2002a; Stoev 2008b) as well as by some secondary bacterial infections as a result of pronounced immunosuppression in the affected animals (Stoev et al. 2000a, b; Oswald et al. 2003, 2005).

Among the mycotoxins, mainly ochratoxin A (OTA), citrinin (CIT) and fumonisin B_1 (FB_1) are reported to have nephrotoxic effects and to be responsible for nephropathy problems in various countries (Voss et al. 2001; Stoev 2008a, b; Stoev et al. 1998a). Fumonisins are produced by *Fusarium verticillioides (F. moniliforme), F. proliferatum* and other *Fusarium* species contaminating mainly corn (Dutton and Kinsey 1995), whereas OTA is mainly produced by *Aspergillus ochraceus* and *Penicillium viridicatum,* which are encountered in feeds/foods stored for a long period of time. Penicillic acid (PA) is another mycotoxin produced by *P. aurantiogriseum* strains and some other fungi such as those of the *Aspergillus ochraceus* group, which are the major producers of OTA in the warmer climatic zones (Stoev et al. 1998a; Stoev 2008a). The same mycotoxin was found to be also involved in mycotoxic nephropathy in animals (Stoev et al. 2001, 2002a, b, c). CIT is usually produced by *P. citrinum,* which can also be considered as a storage fungus. However, the data for the combined exposure to OTA, CIT, FB_1 and PA, which might spontaneously occur under field conditions (Stoev et al. 2002a; Stoev 2008b), are very scarce and incomplete.

The amount of OTA in South African feeds (70–80 μg/kg) from farms with nephropathy problems were significantly less than that presented as the explanation for Danish mycotoxic porcine nephropathy (MPN) (Krogh 1976; Krogh et al. 1974). It seems, therefore, that South African porcine nephropathy may have a multitoxic aetiology because it cannot be explained by the concentration of OTA alone, and the conventional explanation appropriate for northern latitudes does not seem to apply.

On the other hand, the mixtures of mycotoxins usually have at least an additive, if not synergistic, toxic effect. The multiple mycotoxins contamination in various feeds presents new concerns since the data on the effects of simultaneous exposure is still very scarce. There are only a few in vitro or in vivo studies which can help us to predict the outcome (Klaric et al. 2007; Kubena et al. 1997; Stoev 2008a, b). In addition, some studies with pigs and chicks clearly showed an increase in the toxicity of OTA when it is ingested together with PA (Micco et al. 1991; Stoev et al. 2001, 2004). A similar synergistic toxic effect between OTA and PA is reported in mice (Sansing et al. 1976; Shepherd et al. 1981). Some studies suggested an important role of the pancreatic enzyme carboxypeptidase A in the partial detoxification of OTA in the small intestine in rats (Doster and Sinnhuber 1972; Suzuki et al. 1977). Parker et al.(1982) showed that PA inhibits carboxypeptidase activity both in vitro and in vivo, and such inhibition may significantly impair the primary detoxification of OTA in the intestinal tract and so be partly responsible for the enhanced toxicity of OTA when ingested together with PA. The hepatobiliary excretory dysfunction, which can be induced by PA (Chan and Hayes 1981), may also result in decreased hepatobiliary excretion of OTA. Such synergism between OTA and other mycotoxins (such as PA, CIT or FB_1) under field conditions may be responsible for the spontaneous MPN in South Africa, which is associated with relatively low contamination levels of OTA in feed. A similar nephropathy caused by the same combination of mycotoxins has been recently observed in Bulgaria (Stoev et al. 2009b). The low contamination levels of OTA in forage fed in South Africa suggest a possible synergistic interaction between OTA and other mycotoxins (produced by the same ochratoxinogenic or other fungi), enhancing the nephrotoxicity of OTA and/or having an additional nephrotoxic effect, which remains to be proved.

Having in mind the proven synergistic effects between various mycotoxins, the purpose of this study was to carry out extensive field toxicological and mycological investigations in order to assess the probability of involvement of all possible mycotoxins in the etiology of MPN seen in some South African farms, as well as to identify the main producers of the same mycotoxins. The risk assessment for animals and humans potentially exposed to multi-mycotoxins suffers from a lack of adequate information, because synergistic or antagonistic interaction between various mycotoxins are often seen, but not taken into account when assessing the possible cause of various spontaneous cases of nephropathy.

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Materials and methods

Sampling

Twenty-four feed samples were collected in years 2007 and 2008 (a total of 48 feed samples) from various pig farms in South Africa (Baviaanspoort Abattoir, R&R LTD Abatoir, Porkus Abattoir) having nephropathy problems (enlarged and mottled or pale appearance of kidneys) at slaughter time (Fig. 1). Samples were taken from several locations in the food storehouses (top, middle and bottom) after thorough mixing, and subsequently the joint sample was also mixed. Most of the samples came directly from the farms with nephropathy problems and may represent the actual toxic feed. Other feed samples came from some private houses in South Africa (Limpopo province) having nephropathy problems (enlarged or mottled appearance of kidneys) at slaughter time. Some of the feed samples would not be directly representative of the nephrotoxic feed, because sampling was always retrospective. The feed samples were then frozen at −20°C until analysis.

Ten blood samples were also taken in 2007 and 2008 (a total of 20 serum samples) at slaughter time from 20 pigs having nephropathy problems and originating from three of the same farms. After coagulation of the blood, the serum was separated and the serum samples were then frozen at −20°C until toxicological analysis.

Histological examination

Materials for histological examination were taken from 40 kidneys originating from the same farms and showing “mottled”, “enlarged and mottled” or “pale” appearance of kidneys at slaughter time in the years 2007 and 2008. Kidney samples were fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin wax, sectioned at 6 μm and stained with hematoxylin-eosin. Periodic acid-Schiff (PAS) stain was also used for proving of lipoproteid, glycoproteid or mucoproteid substances and especially for proving the thickening of basement tubular membranes (with lipoprotein structure). Some materials were stained according to Weigert with iron haematoxylin for proving the presence or absence of fibrin in various cyst formations. Kidney and liver sections from the same pigs were also silver-stained specifically for evidence of leptospirosis.

Fig. 1 Macroscopic appearance of kidneys with mycotoxic porcine nephropathy (MPN). Enlargement and mottled appearance of group 2 kidneys in pigs aged 6–8 months

Mycotoxin extraction from feed samples and clean-up procedures

A multi-mycotoxin extraction method (multi-mycotoxin screen), which is similar to that of Patterson and Roberts (1979), was used to analyse the feed samples from farms or private houses with nephropathy problems. In the same method, two extracts can be generated in one step: a neutral fraction (containing mainly: aflatoxins-B1, B2, G1, G2 and M1; trichothecenes-T-2 toxin, diacetoxyscirpenol, deoxynivalenol, fusarenon X, nivalenol and their acetyl derivatives; zearalenone; patulin; sterigmatocystin; unknown metabolite) and an acid fraction (containing mainly: citrinin, ochratoxin A, kojic acid, cyclopiazonic acid, penicillic acid). Most of the mycotoxins of interest (ochratoxin A, citrinin, penicillic acid, penitrem A, deoxynivalenol and zearalenone) were extracted via the same method. In this case, 25 g of milled sample was weighed into a conical flask and 100 ml acetonitrile/H_2O (9:1, v/v) was added and placed on a mechanical shaker for 1 h. The content was filtered through a Whatman no 2 V filter paper into a separation funnel and the filtrate was defatted twice with 25 ml iso-octane. Thirty ml saturated Na_2CO_3 solution diluted to 50 ml was added into the defatted filtrate and the extraction was performed (three times) with 25 ml of dichloromethane through a bed of anhydrous Na_2SO_4 into a round-bottom flask. The supernatant aqueous layer was retained for the acid phase, whereas the dichloromethane extract was dried by rotary evaporation, reconstituted with 2 ml acetonitrile and transferred into a previously soaked dialysis tube with a knot tied at one end to form a sac. After emptying the extract into the dialysis, a knot was tied on the other end and then placed into a boiling tube containing 40 ml 30% acetone, sealed with parafilm and placed on a shaker overnight. The overnight dialysate was then transferred to a separation funnel and extracted three times with dichloromethane and passed through a bed of Na_2SO_4 anhydrous as already described. The extract (neutral fraction) was then reconstituted with 2 ml dichloromethane and the content put into a 0.5-ml screw-cap vial, dried by passing through a stream of N_2 gas and stored at 4°C for

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further analyses. Regarding the acid phase (acid fraction), 50 ml 1 M H_2SO_4 was added gently and shaken, and allowed to stand until the effervescence has subsided. The extraction was performed with 25 ml dichloromethane (×3 times) through a bed of Na_2SO_4 anhydrous into a round-bottom flask. The filtrate was dried by rotary evaporation. The content was then reconstituted with 2 ml dichloromethane, put into a 0.5-ml screw-cap vial and dried by passing through a stream of N_2 gas and stored at 4°C for further analyses.

Extraction and clean-up of FB_1 was performed by a separate procedure, because fumonisins are only soluble in lower alcohols and acetonitrile and therefore require an additional extraction step compared to other mycotoxins. The extraction and clean-up of fumonisins was performed according to Hinojo et al. (2006) using a SAX column. Ten grams of milled sample was weighed and extracted with 20 ml CH_3OH/H_2O (60:40, v/v) (after shaking for 1 h) through a Whatman no. 2 V filter paper. Ten ml of the filtrate was cleaned by passing it through a SAX cartridge previously conditioned with 5 ml each of CH_3OH and CH_3OH/H_2O (3:1, v/v). The cartridge was washed with 8 ml of CH_3OH/H_2O (3:1, v/v) and 3 ml of CH_3OH. Flow rate was maintained at 1 ml/min. The content containing FB (P-fraction) was then flushed with 10 ml of 1% acetic acid in CH_3OH into a screw-cap vial, evaporated by passing through a stream of N_2 gas and stored at 4°C until analysed.

TLC analysis of feed extracts

TLC (thin layer chromatography) analysis was performed according to Patterson and Roberts (1979). A two-dimensional thin layer chromatographic technique was used as 20 ml of the neutral and acid fractions (dissolved in an appropriate solvent-mainly dichloromethane) obtained from each feed sample were spotted on TLC plates (about 1 cm from the edge of a silica gel TLC plate) and dried in a warm stream of air. The spotted plates were then developed in TLC tanks using two solvents (mainly CEI and TEF for most of the mycotoxins; CM2 and BWA for FB_1 or MON, CtE and ChE for ZEA, etc)[1] in two-dimensional directions and were dried after each development. The solvents move the toxins to the solvent front. The same procedure was followed for the standard mycotoxins. The plates were visualized under ultraviolet (UV) light at 254 and 365 nm for the presence of any fluorescent or absorbing spot and were compared with the standard plates for each analysed mycotoxin. The identity of the mycotoxins was confirmed as the plates were sprayed with a specific reagent for a particular mycotoxin, such as *p*-anisaldehyde for FB_1, NH_3 vapour for PA, diazotised benzidine for ZEA, chromotropic acid for DON and other trichotecenes, Pauly's reagent for KA or PAT, Ehrlich's reagent for CA, etc. The R_F value for each spot was calculated and compared with the $R_{F.}$ value of a standard for each mycotoxin.

A new metabolite (UM-unknown metabolite) with green fluorescence (Fig. 2), which was found to differ from known mycotoxins and which was seen in many feed samples, was purified by repeated one-dimensional preparative TLC with alternating mobile phases (CEI and CM). Silica containing the unidentified metabolite was scraped from the plate into a flask, dissolved in acetone, filtered through Whatman No. 1 filter paper and dried with a rotory evaporator. The residues were reconstituted with dichloromethane, dried by a stream of N_2 gas and then analysed. In order to prepare a sufficient quantity of UM, which was found to be produced mainly by *P. polonicum,*, cultivation of the same species on solid medium (shredded wheat) and liquid medium (YES medium) and extraction by the procedure mentioned above was performed. The mass spectrum of the purified extract of the UM was determined by liquid chromatography/mass spectrophotometry (LC/MS) using Synapt HDMS Time-of-Flight mass spectrometer system equipped with Acquity-UPLC™ Sample Manager, Sample Organizer and Photodiode Array (PDA) UV detector (Waters, Milford, USA) as described recently (Stoev et al. 2009b).

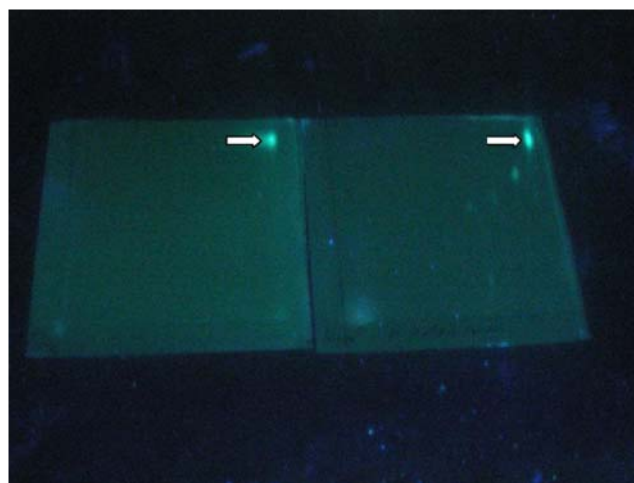

Fig. 2 Green fluorescence of a new secondary fungal metabolite, which was seen in many feed extracts and was found to differ from all known mycotoxins by its fluorescence, RF values, ultraviolet light spectrum and molecular mass

[1] CEI - Chloroform-Ethyl Acetate-Propan-2-ol (90:5:5, v/v/v);
TEF - Toluene-Ethyl Acetate-Formic Acid (6:3:1, v/v/v);
CM - Chloroform-Methanol (95:5, v/v);
CM2 - Chloroform-Methanol (3:2 ,v/v);
BWA - Butanol-Water-Acetic acid (12:5:3, v/v/v);
CtE - Carbon tetrachloride-Ethanol (98:2, v/v);
ChE - Cyclohexane-Ether (3:1, v/v)

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HPLC analysis of feed extracts and serum samples

All mycotoxins of interest found by TLC in the feed samples were then quantified using HPLC. HPLC analysis was performed using a Shimadzu system (Kyoto, Japan), consisting of liquid chromatograph LC 20A fitted to degasser DGU 20A3, auto sampler (injection) SIL 20A, communications bus module CBM 20A, column oven CTO 20A, photodiode array detector SPD M20A and fluorescence detector RF 10AXL, all connected to a gigabyte computer with Intel Core DUO with Microsoft XP. For HPLC analysis of PA, CIT, OTA, PenA and DON, photodiode array (PDA) detector SPD M20A with Waters Symetry C18 column (250 mm long, 4.6 mm internal diameter) were used with appropriate mobile phase, flow rate and running time for each mycotoxin, as described by Kokkonen et al. (2005) or Abdulkadar et al. (2004) with some modifications. Reagents and HPLC conditions for the same mycotoxins were: mobile phase—acetonitrile-water (50:50, v/v); flow rate—0.8 ml/min; injection volume—10 µl per sample; running time—30 min; column temperature—30°C. The differences in HPLC conditions for DON were: mobile phase—acetonitrile-water (15;85, v/v); flow rate—1 ml/min, running time—20 min. The detection of the mycotoxins was accomplished at the maximum wavelength of each mycotoxin as PDA setting was usually between 190 and 331 nm. The identification of the mycotoxins was based on the comparison of the UV-spectra and the retention times of the detected peaks with those of the standard substances. Mycotoxins were quantified using peak area and external standard calibration. The calibration curves for all investigated mycotoxins were found to be appropriate for their validation. The detection limits ranged usually from 0.02–0.03 µg/kg for OTA up to 10–15 µg/kg for DON and FB1. Selected feed samples (free of mycotoxins) were spiked with known levels of mycotoxins standards and were processed in a similar manner as the other samples in order to establish recovery rates for various mycotoxins. Generally, the recovery of the investigated mycotoxins was found to be above 60%, but most often between 85 and 95%, whereas for FB_1 it was above 90%. Data received in this study were not adjusted based on the recovery rates obtained.

For performing of FB_1 analysis, RF-10AXL fluorescence detector (FD) was used with the same column and respective wavelength (excitation and emission), mobile phase, flow rate and running time. The settings of the FD were: wavelength of 420 nm excitation and 500 nm emission. Reagents and HPLC conditions were: mobile phase—acetonitrile-water in the ratio 60:40; flow rate—1 ml/min; injection volume—10 µl per sample; running time—30 min; column temperature—30°C. Derivatization reagent used for FB_1 was naphthalene dicarboxyaldehyde (NDA), and HPLC was performed according to Bennett and Richard (1994). Residues (sample and standards) were dissolved with 1 ml methanol into 100 µl of sample or mycotoxin standard dissolved with methanol in the HPLC vial. The following reagents were added in sequence and mixed after each addition: 100 µl 0.05 M sodium borate buffer (pH 9.5), 50 µl sodium cyanide and 50 µl NDA. This was followed by incubation in a water bath set at 50°C for 20 min, after which the samples were cooled and diluted with 700 µl 0.05 M phosphate buffer (pH 7) acetonitrile (40:60, v/v).

Extraction and HPLC analyses of DON from serum samples were performed according to Bily et al. (2004), and those of CIT according to Phillips et al. (1980). PA in serum was extracted and analysed according to Hanna et al. (1981). OTA in serum was extracted and analysed according to Boudra and Morgavi (2006). The same methods usually concern only the free fractions of the mycotoxins investigated, but disregard the conjugates.

Fungal screening and identification

Fungal screening and identification of mycotoxins in analysed feed samples was performed by the following mycological analytical procedures: fungal isolation on potato dextrose agar (PDA) and Ohio Agricultural and Experimental Station agar (OAEA), sub-culturing on PDA, malt extract agar (MEA) and Czapek yeast extract agar (CYA), and macro- and microscopic identification. The final step involved: (1) DNA extraction, (1) PCR (polymerase chain reaction) amplification, (3) purification of PCR product, (4) product quantification, and (5) DNA sequence for a confirmation of various species of fungi.

A serial dilution technique was employed for each feed sample as 1 g of the sample was diluted in 10 ml ringer solution and vortexed, and subsequently 1 ml of this suspension was transferred to 10 ml Ringer solution and vortexed, etc. One ml of each suspension was then aseptically inoculated on PDA and OAEA in Petri dishes and incubated at 25°C for 7–14 days. The culture method employed is that suggested by Klich (2002). From the 4th to the 7th day, plates were screened for different types of fungal colonies, and counted using a colony counter. After incubation, the number of fungal colonies per gram of sample was calculated and expressed in colony forming units per gram (cfu/g). For the identification of fungal species, CYA, MEA and PDA were used. The hyphae and conidia from each colony representing each fungal species were transfered aseptically onto three spots on PDA and incubated at 30°C for 7–14 days for further identification. Determination of each species of fungi was done using the keys of Klich and Pitt (1988) and Klich (2002) for *Aspergillus* spp., Pitt and Hocking (1997) for *Penicillium*

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449 and Nelson et al. (1983) for *Fusarium* spp. This was done by observing both macroscopic characteristics of the colonies on various media used as well as the microscopic morphology and measurements of the conidiophores (after staining mycelia with 0.1% fuchsin dissolved in lactic acid or with lactophenol blue solution). The isolates were stored at 4°C for further uses and PCR analysis.

When the morphological characteristics of individual fungal species were not sufficient for clear identification, further analysis was performed. All isolated fungi were further identified via PCR analysis. The mycelia for PCR analysis were scraped and transferred into a 0.5-ml sterile screw-cap vial containing 200 µl of Ringer solution, freeze-dried and stored at −40°C until analysed. Fresh mycelia of some fungi were also used for PCR analysis in some cases. The technique involving the comparison of nucleic acid profiles of individual fungal species was employed using an automated sequencer. DNA extraction, PCR amplification, purification and quantification of PCR product, and DNA sequencing and analysis were performed using similar technologies to those described by Samson et al. (2004) and Geiser et al. (2004) with some modifications, using Invisorb Spin Plant Mini Kit (Invitek, Berlin) for DNA extractions from plant material (fresh, frozen or dried materials) in addition to MSB® Spin PCRapace for ultrafast purification and concentration of PCR-fragments (Invitek). This PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 owned by Hoffmann-LaRoche. Samples were further analysed on an ABI PRISM 3700 Genetic analyzer (AB; Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). The forward and reverse sequences of the PCR products were assembled with a DYEamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Roosendaal, Netherlands) using the programmes SeqMan and EditSeq from the LaserGene package (DNAStar, Madison, WI). The PCR analysis was performed in Inqaba Biotec in Pretoria (South Africa) as an Inqaba Finch server was used for DNA sequencing and identification of fungi.

Determination of mycotoxinogenic potentials of isolates

Fungal species were further analysed for their capability to produce various mycotoxins. The isolates were individually cultured on YES agar in Petri dishes and incubated at 25°C for 2–3 weeks according to the method of Singh et al. (1991). A TLC technique was then employed whereby 5 g of isolate including the medium was plugged and dissolved in 10 ml of dichloromethane. This solution was further filtered, and the filtrate put in a screw-cap vial and dried under a stream of N_2 gas then stored at 4°C until analysed. Two-dimensional TLC (Patterson and Roberts 1979) was employed for the detection of mycotoxins. The identity of the mycotoxins was confirmed as the plates were sprayed with a specific reagent for a particular mycotoxin as described above and the R_F value for each spot was calculated and compared with the R_F. value of a standard for each mycotoxin.

Chemicals and mycotoxins

The standards of all analysed mycotoxins were obtained from Sigma Bioscience (St Louis, USA) or Merck, except for penicillic acid (PA) which was obtained from A.G. Scientific (San Diego, CA, USA).

All solvents for HPLC analysis were HPLC grade and were obtained from Merck, Darmstadt, Germany. All chemicals for PCR analysis were purchased from Invitek.

Statistical analysis

The Student's *t* test was used to calculate the mean values and standard error of the mean of various parameters as appropriate.

Results

This nephropathy ("mottled", "enlarged and mottled" or "pale" appearance of kidneys) was established during the meat inspection at slaughter time (Fig. 1). As a whole, the frequency and duration of the observed nephropathy in different batches of slaughtered pigs varied significantly. According to their macroscopic appearance, kidneys could be grouped into three different groups: mottled, enlarged and mottled (marbled) or enlarged and pale (Table 1). These groups can be considered to illustrate the progressive stages of development of the disease.

Group 1 kidneys were no more than slightly enlarged and were sometimes slightly paler (grey-brown) than normal. On their surface, there were occasional or numerous grey-white foci the size of which varied from scarcely visible to 1–2 cm in diameter. The density of kidneys, assessed by palpation, was normal or slightly increased. On the cut surface, the pale foci were confined to the cortex and were often larger in the deep part of the cortex. They looked like pale-grey strips of variable width orientated radially to the renal pelvis. The pathomorphological picture was dominated by two types of changes: degenerative, affecting mainly the proximal tubular epithelial cells, and proliferative, characteristic by limited proliferation of connective tissue and infiltration of mononuclear cells in the interstitium. Initially, granular degeneration (Fig. 3) and sometimes hyaline droplet (Fig. 4) or vesicular degeneration, was mainly found in kidneys with scarce grey-white foci on their surface. Necrosis and desquamation of the affected epithelium was also observed. The proximal

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t1.1 **Table 1** Classification of kidneys with spontaneous MPN in South Africa according to macroscopic and histological changes, listed in chronological order of disease development

Group	Kidney characteristics	Enlargement by volume (%)	Number of cases	Frequency (%)
1	Mottled	Up to 20	25	62.5
2	Enlarged and mottled	20–100	12	30.0
3	Enlarged and pale	50–200	3	7.5

tubules were often dilated and their lumen contained necrotic debris and granular or hyaline casts. In the glomeruli, there were no pronounced changes and only dilatation of the capsular space, which contained serous liquid, was rarely observed. The interstitium was usually slightly affected by a focal oedema and a limited focal growth of fibroblastic tissue and mononuclear cells infiltration around the damaged tubules (Fig. 5). The changes in kidneys at this stage were characteristic of an acute developing nephrosis. As the disease advanced, the quantity of fibroblastic connective tissue and mononuclear cells (lymphocytes, monocytes, plasmatic cells and eosinophilic leucocytes) increased. As a rule, the mononuclear cell proliferation always preceded that of connective tissue.

Group 2 kidneys (enlarged and mottled) bore many diffuse grey-white foci which were often confluent and spread over a large part of the surface giving a mottled and even marbled appearance (Fig. 1, Table 1). The foci were usually seen to be confined to the cortex and rarely penetrated into the medulla. The kidneys were slightly more dense than normal. Sometimes, adhesions between the capsule and the parenchyma were observed. The pathomorphological picture was dominated by degenerative or atrophic changes affecting the proximal tubules as well as by proliferative changes in the interstitium. However, the interrelation between these two types of change varied widely. There was a marked increase of proliferation of the fibroblastic connective tissue and infiltration of mononuclear cells in comparison to those in group 1 kidneys. In some kidneys, activation of vascular and capillary endothelium was also observed. Lymphatic cysts containing serous fluid and lined by endothelium were often evident. Sometimes, small petechial haemorrhages were seen under the capsule.

Group 3 kidneys were significantly enlarged, pale (Table 1) and markedly more dense. These kidneys were diffusely grey-white and sometimes their surface was slightly undulating. The pathomorphological picture was dominated by markedly increased proliferation of connective tissue and reduction of the amount of mononuclear cells in comparison to group 2 (Fig. 6), so that the normal structure of the kidneys was almost obscured. In one case, a fibrosis of connective tissue was also observed. The pathomorphological picture was dominated by tubular atrophy (Fig. 6) accompanied by thickened basement membranes and the first signs of hyalinization and sclerosis of glomeruli (Fig. 7). Some tubules had collapsed and were surrounded by connective tissue. Similar, but less pronounced, changes were sometimes observed in the region of the loop of Henle. The capillary endothelium, vascular endothelium and vascular adventitial cells showed activation and proliferation. Lymphatic cysts were sometimes observed in the cortical part of kidneys. Small retention cysts had also formed within the dilated tubules.

The kidney vascular system in some cases in groups 2 and 3 showed an activation of capillary or vascular endothelium and vascular adventitial cells, which had often proliferated

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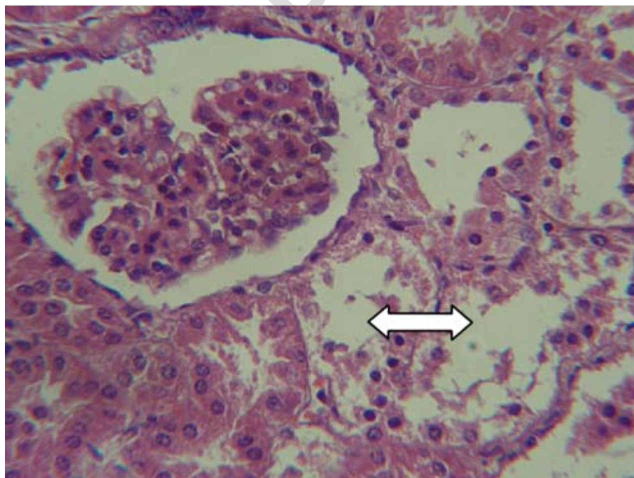

Fig. 3 Group 1 kidney showing granular degeneration in the epithelial cells of proximal tubules. H/E×300

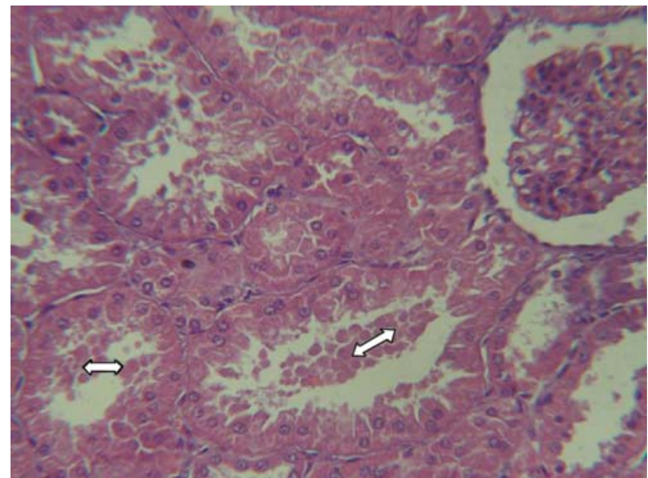

Fig. 4 Group 1 kidney showing hyaline droplet degeneration and granular or hyaline casts in the tubular lumina. H/E×300

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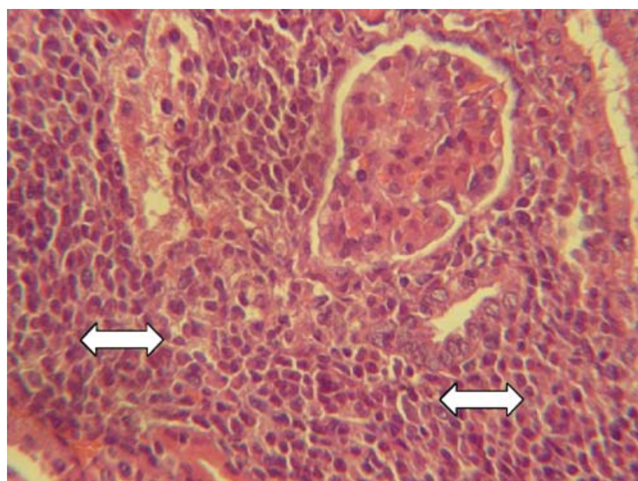

Fig. 5 Group 1 kidney showing limited focal proliferation of fibroblastic tissue and mononuclear cells infiltration around the damaged tubules. H/E×300

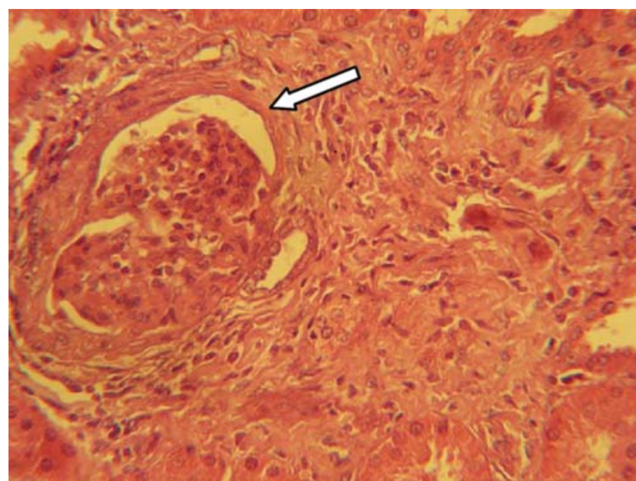

Fig. 7 Group 3 kidney showing focal interstitial fibrosis and initial glomerular sclerosis. H/E×300

602 (Fig. 8). In addition, degenerative changes in the vascular
603 intima and media (Fig. 9), as well as serous fluid in the
604 lumen of Bowman's capsules of glomeruli, telangiectasis and
605 perivascular oedema (Fig. 9), were observed.

606 Pathomorphological investigations of silver-stained kid-
607 ney and liver sections revealed no presence of leptospires.
608 Furthemore, there were no macroscopic changes at slaugh-
609 ter time such as icterus, hepatitis, or meningoencephalitis
610 which are typical for leptospirosis, and no aborted fetuses
611 from pregnant sows in the affected farms, typical for
612 leptospirosis, were seen.

613 Preliminary TLC analysis of feed extracts showed
614 presence of the following mycotoxins in most of the feed
615 samples: OTA, PA, CIT, FB_1, PenA and DON. That is why
616 the same feed extracts were analysed for the same
617 mycotoxins by HPLC, allowing quantification of these
618 mycotoxins in the samples (Table 2). Contamination levels
619 of OTA were comparatively low (67.8 and 75.2 μg/kg) in
620 contrast to high contamination levels of FB_1 (5,289 and
621 5,021 μg/kg) and modest levels of PA (149 and 251 μg/kg).

622 Serum samples from pigs with MPN, originating from
623 MPN-affected farms, also revealed the presence of some of
624 these mycotoxins when analysed via HPLC (Table 3).

625 A new secondary fungal metabolite (UM) with green
626 fluorescence, which was seen to differ from known myco-
627 toxins by its fluorescence and R_F values, was present in 28
628 feed extracts (58.3%) and was found to be produced by a
629 wide variety of species, but mainly by *P. polonicum* (Tables 4
630 and 5). UV spectrum of UM shows that the molecule can be
631 detected at maximum wavelength of 204 nm.

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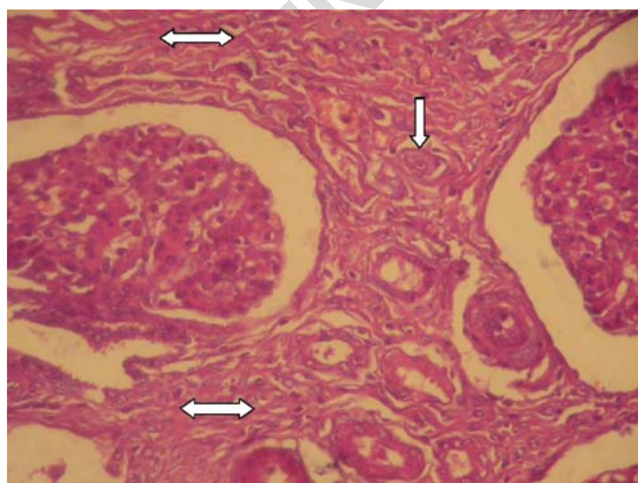

Fig. 6 Group 3 kidney showing increased proliferation of connective tissue and reduction of the amount of mononuclear cells in the interstitium. Slight interstitial fibrosis accompanied by initial tubular atrophy. H/E×300

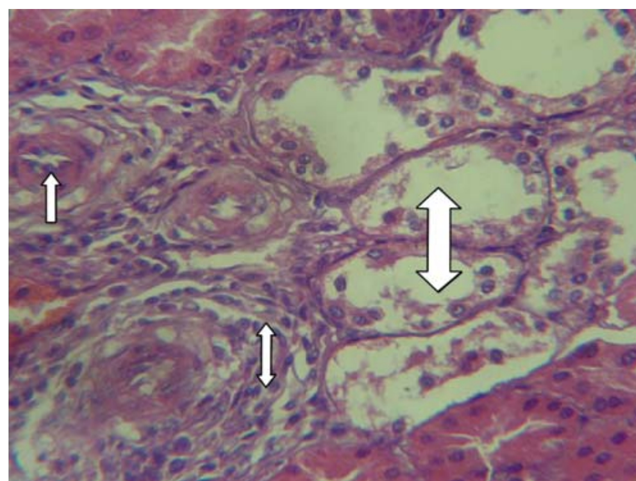

Fig. 8 Group 3 kidney showing an activation of capillary or vascular endothelium and vascular adventitial cells. Granular degeneration in the epithelial cells of proximal tubules. H/E×300

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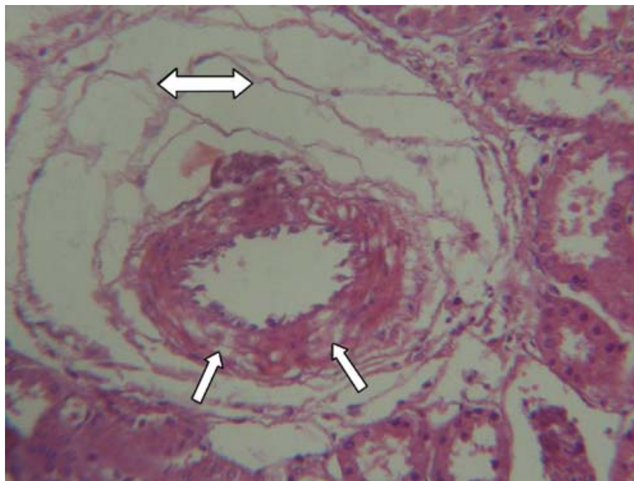

Fig. 9 Group 3 kidney showing degenerative changes in the vascular intima and media, and perivascular oedema. H/E×260

632 A heavy contamination with *Gibberella fujikuroi* var. 633 *moniliformis (Fusarium verticillioides*) was seen in the feed 634 samples and all isolated species were able to produce FB_1. 635 A heavy contamination with *Penicillium aurantiogriseum* 636 complex (mainly *P. polonicum*), which appeared to be a 637 good producer of PA and UM, was also observed in many 638 feed samples (47.9%) coming from pig farms with 639 nephropathy problems in South Africa. Surprisingly, a light 640 contamination with the usual producers of OTA (*A.* 641 *ochraceus, P. verrucosum*) and citrinin (*P. citrinum*) was observed in the same feed samples and these species were 642 isolated as rare component of the mycobiota. Some of the 643 *A. niger, A. wentii*, *P. commune* and *A. fumigatus* species 644 were also found to produce small amounts of OTA 645 (Table 4). A few isolates were also found to produce UM 646 (Table 5). 647

Discussion 648

Mycotoxicological and microbiological investigations con- 649 firmed that nephropathy observed is due to various 650 mycotoxins, encountered in ground, complete feeds for 651 pigs. This report appeared to be the first report for 652 spontaneous cases of MPN in South Africa. 653

Pathomorphological changes and especially vascular 654 damages in kidneys in spontaneous MPN in South Africa 655 resemble much more those in MPN observed in Bulgaria 656 (Stoev et al. 1998a) than in Scandinavia (Krogh 1976). 657 Such vascular damage has not been seen in classical MPN 658 as described in Scandinavian countries or elsewhere. 659 These differences between South African MPN and 660 classical MPN as well as the similarity between South 661 African and Bulgarian MPN could be due to the 662 interaction between OTA and other mycotoxins, especially 663 FB_1, which was supported by microbiological and myco- 664 toxicological investigations on consumed feeds (Stoev et 665 al. 2009b), showing the presence of the same nephrotoxic 666

t2.1 **Table 2** Mean concentrations of various mycotoxins in feed samples (n=24) from pig farms or private houses in South Africa having nephropathy problems (mottled appearance of kidneys) at slaughter time and co-occurrence with the most important mycotoxins

Mycotoxin	Concentration in feed µg/kg (ppb)	Number of positive samples	Co-occurrence with OTA (n)	Co-occurrence with FB_1 (n)	
Year 2007					t2.3
Ochratoxin A (OTA)	67.8±39.2	20		19	t2.4
Penicillic acid (PA)	149±64	10	9	8	t2.5
Citrinin (CIT)	2.7±1.0	17	15	14	t2.6
Fumonisin B1 (FB_1)	5,289±1,034	20	19		t2.7
Deoxynivalenol (DON)	119±60	8	7	6	t2.8
Penitrem A (PenA)	1,976±783	16	14	13	t2.9
Year 2008					t2.10
Ochratoxin A (OTA)	75.2±20.6	21		18	t2.11
Penicillic acid (PA)	251±69	17	16	14	t2.12
Citrinin (CIT)	3.9±1.2	16	15	14	t2.13
Fumonisin B1 (FB_1)	5,021±844	19	18		t2.14
Deoxynivalenol (DON)	87±25	23	21	18	t2.15
Penitrem A (PenA)	1,521±579	17	16	15	t2.16

± SEM (standard error of the mean)

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t3.1 **Table 3** Mean concentration of various mycotoxins in serum samples (n=10) from pig farms in South Africa having nephropathy problems (mottled appearance of kidneys) at slaughter time and co-occurrence with the most important mycotoxins

Mycotoxin	Concentration in serum µg/l	Number of positive samples	Co-occurrence with OTA	Co-occurrence with PA
Year 2007				
Ochratoxin A (OTA)	4.6±3.9	5		5
Penicillic acid (PA)	5.0±4.1	8	5	
Citrinin (CIT)	0.4±0.3	6	4	5
Deoxynivalenol (DON)	1.5±1.5	1	0	1
Penitrem A (PenA)	6.5±6.4	2	1	1
Year 2008				
Ochratoxin A (OTA)	0.9±0.8	4		3
Penicillic acid (PA)	4.7±3.4	4	3	
Citrinin (CIT)	0.4±0.3	4	2	2
Deoxynivalenol (DON)	0	0	0	0
Penitrem A (PenA)	10.6±10.5	2	0	0

± SEM (standard error of the mean)

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mycotoxins and the respective fungi, but at different levels, in both countries.

FB_1 may also contribute to the immunosuppressive effect of OTA and a complication of the pathomorphological picture by increasing secondary bacterial infections in pigs with spontaneous MPN (Stoev et al. 2000b; Stoev 2008a, b). That is suggested by the observed increase of intestinal colonization by pathogenic *E. coli* in FB_1-treated pigs as reported by Oswald et al. 2003.

Some previous studies confirmed that the m/z of UM does not correspond with those of the penitrems provided by Kyriakidis et al. (1981) and metabolites of other *Penicillium* cultures provided by Smedsgaard and Frisvad (1996), Smedsgaard (1997) and Smedsgaard and Nielsen (2005). Therefore, the novel mycotoxin cannot be assigned to any known metabolite reported in the literature (Stoev et al. 2009b). Nuclear magnetic resonance (NMR) analysis remains to be performed in order to determine the structure of UM.

The low presence of some mycotoxins in the serum samples, unlike the feed samples, could be due to the lack of a direct connection between the feed samples and blood samples, because the feed sampling is always retrospective and sometimes the feed sources of the affected farms have been recently changed. On the other hand, only the free fractions of mycotoxins were investigated. In addition, feed deprivation of pigs before slaughtering could eliminate some mycotoxins from the blood. For example, fumonisins have a poor absorption and a rapid excretion mainly through the faeces (via the liver) rather than the urine, and it is unusual to discover these mycotoxins in pig blood (Taranu et al. 2008), especially after the feed deprivation of pigs before slaughtering. Therefore, the study of FB_1 in the blood was omitted. However, we should take into account that the same mycotoxin undergoes an enterohepatic recirculation, which could slow up its elimination from the organism of animals (Voss et al. 2007).

In addition, it is difficult to evaluate the content of various mycotoxins in the ground feeds because the fungi invade only a minor fraction of feed particles with appropriate conditions for a growth of fungi, which explains why mycotoxin contamination of feedstuffs in two nearby places is often completely different. That could be the reason that some of the mycotoxins were found only in a small number of blood samples of pigs from the same farm.

The overall concentration of OTA in feed samples was definitely lower than the levels of 200–1,000 µg/kg required to reproduce MPN of similar severity (Krogh 1976; Stoev et al. 1998a,b, 2002b,c). Therefore, it can be concluded that the MPN in South Africa may have a multitoxin or multifactor aetiology, because it cannot be explained by the concentration of OTA alone and the respective OTA-producing fungi (usually *P. verrucosum* and *A. ochraceus*) found in the feeds suspected to cause the nephropathy problems. This conclusion is in good agreement with the present investigations, which showed high mean contamination levels of FB_1 (5,021–5,289 µg/kg) in South African feed samples from farms with nephropathy problems and comparatively low levels of OTA (67–75 µg/kg), CIT (2.7–3.9 µg/kg) and PA (149–251 µg/kg) in the same feeds. It is also important to mention that the percent

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t4.1 **Table 4** Percentage of contaminated feed samples (24 samples for 2007 and 24 samples for 2008) with *Fusarium*, *Penicillium* and *Aspergillus* fungi, level of fungal contamination, number of strains isolated and number of toxinogenic strains isolated as well as kind of mycotoxins produced by each fungal strain and co-occurrence of mycotoxins with OTA and FB_1 in various feed samples from pig farms or private houses in South Africa having nephropathy problems (enlarged and mottled appearance of kidneys) at slaughter time

	Strain isolated	Percentage of contaminated samples (%)	Range of fungal contamination (cpu/g)	Number of strains isolated	Number of toxinogenic strains	Kind of mycotoxins produced (number of species)
t4.3	*Fusarium* species					
t4.4	*Gibberella fujikuroi (var. moniliformis)*	62.5	2×10^3–8×10^6	30	30	FB_1(15), FB_2(4)[b], ZEA (12)[b], MON(4), UM(1)
t4.5	*F. verticillioides (F. moniliforme)*	2	4×10^6	1	1	FB_1(1), ZEA(1)[b]
t4.6	*F. oxysporum*	2	3×10^4	1	1	ZEA(1)
t4.7	*F. graminearum*	4.2	3×10^3–4×10^5	2	2	ZEA(2), DON(2), T-2 (1), DAS(1)
t4.8	*Penicillium* species					
t4.9	*P. viridicatum (P. verrucosum)*	2	5×10^3	1	1	OTA(1), CIT(1)[a], PA(1)[a]
t4.10						XA(1)[a], UM(1)[a]
t4.11	*P. commune*	6.2	4×10^3–5×10^6	3	2	OTA(2), UM (2)[a]
t4.12	*P. citrinum*	6.2	3×10^3–5×10^5	3	3	CIT(3), KA(1), UM(1)
t4.13	*P. expansum*	2	3×10^5	1	1	CIT(1), PAT(1), UM(1)
t4.14	*P. aurantiogriseum*	8.3	3×10^3–4×10^6	4	2	PA(2), UM(1)
t4.15	*P. polonicum*	47.9	5×10^3–6×10^6	23	18	PA (16), UM (18)
t4.16	*P. freii*	2	4×10^3	1	1	PA(1), UM(1)
t4.17	*P. chrysogenum*	4.2	3×10^3–4×10^6	2	1	PA(2), UM(1)
t4.18	*P. crustosum*	6.2	3×10^3–8×10^5	3	3	PA(3), PenA(2), UM(2)
t4.19	*P. simplicissimum*	2	3×10^4	1	1	PA(1)
t4.20	*P. islandicum*	2	5×10^3	1	1	ERY(1), LUT(1), ISL(1), UM(1)
t4.21	*Aspergillus* species					
t4.22	*A. ochraceus*	4.2	2×10^3–3×10^4	2	1	OTA(1), PA(1)[a]
t4.23	*A. niger*	25	4×10^3–3×10^6	12	5	OTA(5), OTC(1)[a]
t4.24	A. wentii	2	3×10^4	1	1	OTA(1), KA(1)[a]
t4.25	*A. candidus*	18.7	4×10^3–8×10^5	9	7	CIT(1), KA(1), UM(7)
t4.26	*A. fumigatus*	45.8	3×10^3–7×10^6	22	22	OTA(3), GLI(2),
t4.27						KA(2), UM(17)[a]
t4.28	*A. flavus*	52	2×10^3–4×10^6	25	25	Afs (21), CA (12), KA(14), UM(12)
t4.29	*A. oryzae*	2	4×10^4	1	1	CA(1), KA(1)
t4.30	*A. restrictus*	2	4×10^4	1	1	UM(1)
t4.31	*Eurotium amstelodami*	8.3	4×10^3–2×10^5	4	1	UM(1)

AFs Aflatoxins, *CIT* citrinin, *CA* cyclopiazonic acid, *DAS* diacetoxyscirpenol, *DON* deoxynivalenol, *ERY* erythroskyrin, FB_1 fumonisin B_1, FB_2 fumonisin B_2, *GLI* gliotoxin, *ISL* islandiotoxin, *KA* kojic acid, *LUT* luteoskyirin, *MON* moniliformin, *UM* unknown metabolite, *OTA* ochratoxin A, *OTC* ochratoxin C, *PenA* penitrem A, *PAT* patulin, *PA* penicillic acid, *T-2* T-2 toxin, *XA* xanthomegnin, *ZEA* zearalenone

[a] Co-occurrence with OTA

[b] Co-occurrence with FB_1

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729 of feed contaminated with the mycotoxins involved was 730 constantly high, above 70%. A similar multimycotoxin 731 etiology was also found for Bulgarian MPN as the same 732 mixture of mycotoxins (188–376 μg/kg OTA with 100% positives; 54–120 μg/kg CIT with 92–96% positives; 838– 733 904 μg/kg PA with 88–92% positives; 3,254–5,564 μg/kg 734 FB_1 with 92–96% positives) was established in Bulgarian 735 feed samples from pig farms with nephropathy problems 736

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t5.1 **Table 5** Percentage of contaminated feed samples (24 samples for 2007 and 24 samples for 2008) with various other fungi, level of fungal contamination, number of strains isolated and number of toxinogenic strains isolated as well as kind of mycotoxins produced by each fungal strain in various feed samples from pig farms or private houses in South Africa having nephropathy problems (enlarged and mottled appearance of kidneys) at slaughter time

	Strain isolated	Percentage of contaminated samples (%)	Range of fungal contamination (cpu/g)	Number of strains isolated	Number of toxinogenic strains	Kind of mycotoxins produced (number of species)
t5.3	*Acremonium strictum*	12.5	4×10^3–5×10^5	6	1	OOS (1)
t5.4	*Pichia guilliermondii*	2	3×10^4	1	–	–
t5.5	*Pichia caribbica*	14.5	3×10^3–5×10^4	7	–	–
t5.6	*Paecilomyces variotii*	8.3	3×10^3–4×10^4	4	–	–
t5.7	*Paecilomyces sinensis*	4.2	3×10^3–2×10^5	2	1	UM (1)
t5.8	*Cladosp. cladosporioides*	4.2	3×10^3–4×10^4	2	–	–
t5.9	*Talaromyces emersonii*	4.2	3×10^3–3×10^4	2	–	–
t5.10	*Cordiceps bassiana*	2	4×10^4	1	–	–
t5.11	*Mucor racemosus*	4.2	3×10^3–2×10^5	2	1	UM (1)
t5.12	*Mucor circinelloides*	10.4	3×10^3–2×10^5	5	2	UM (2)
t5.13	*Candida albicans*	4.2	3×10^3–5×10^4	2	–	–
t5.14	*Candida tropicalis*	6.2	4×10^3–7×10^4	3	–	–
t5.15	*Geomithia pallida*	4.2	5×10^3–8×10^4	2	1	UM (1)
t5.16	*Sacharomyces cerevisiae*	2	7×10^4	1	–	–
t5.17	*Rhodotorula mucilaginosa*	2	5×10^4	1	–	–

OOS Oosporein (isoospoin)

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(Stoev 2008b). The levels of OTA and PA, considered to be the main mycotoxins involved in this nephropathy, were 2–3 times higher as should be anticipated from the higher severity and spread of the same nephropathy in Bulgaria unlike in South Africa (Stoev et al. 2009b).

A possible model for the South African nephropathy could be the renal disease that was previously investigated in depth in Denmark in the 1970s and shown conclusively to be caused by nephrotoxins of fungal origin (mainly OTA and CIT produced by *P. verrucosum* growing on poorly stored feed or grain). However, in the South African nephropathy, similar to the Bulgarian nephropathy, the incidence of OTA measured in the diet and in the serum was much less than should be necessary (according to extensive experimentation in Denmark) to fully account for the renal changes (Stoev et al. 1998a,b). A classification was made of the kidney damage in pigs in Bulgaria. According to the different macroscopic and microscopic changes as well as according to the different stages of the progress of this nephropathy, the kidneys were classified into five separated groups: “mottled kidneys”, “enlarged and marbled kidneys”, “enlarged pale kidneys”, “cystic kidneys” and “fibrotic kidneys” (Stoev et al. 1998a). The pathology in the last two groups of kidneys is not characteristic for nephropathy in South Africa as was seen in the present study. These differences in macroscopical or microscopical picture of the kidneys were considered to be due to the differences in the length of the time of exposure to the mouldy feed (Stoev et al. 1998a) as well as to the differences in the amount or combination of nephrotoxic mycotoxins ingested by each pig.

It seems that some differences between classic Danish porcine nephropathy and South African or Bulgarian nephropathy in pigs (e.g. vascular and edematous changes) as described recently by Stoev et al. (1998a) are probably a result of the effects of other nephrotoxic metabolites, such as FB_1 and PA in addition to the toxic effect of OTA, or may be attributed to synergistic effects between OTA and

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other mycotoxins (Stoev 2008b). Therefore, it is important to understand whether there is a synergistic effect between OTA, CIT, PA and FB_1 in vitro" or in vivo on kidney.

OTA and FB_1 were reported to induce in vitro and in vivo degenerative and apoptotic changes in rat kidney (Petrik et al. 2003). A synergistic effect between OTA and FB_1 was seen in vitro (Klaric et al. 2007; Creppy et al. 2004) as well as in vivo (Kubena et al. 1997). That synergism between FB_1 and OTA could be due to the ability of both toxins to impair protein synthesis and to increase lipid peroxidation, producing reactive oxygen species (Creppy et al. 1984; Rahimtula et al. 1988; Abado-Becognee et al. 1998).

On the other hand, the DNA damage provoked by the combined treatment with OTA and FB_1, measured either by the standard comet assay or Fpg-modified comet assay, showed a synergistic increase in kidney cells in vivo as indicated by the tail length, tail intensity and OTM (olive tail moment), even at low doses which correspond to the daily human exposure (Domijan et al. 2006).

A similar synergistic effect between PA and CIT was reported by Lillehoj and Ciegler (1975), who found that PA and CIT were innocuous when administered alone, but were 100% lethal when given in combination.

Some other reports for a synergistic effect between OTA and CIT have also been made. A synergistic effect has been seen between OTA and CIT in the suppression of concavalin A-induced proliferation of porcine lymphocytes (Bernhoft et al. 2004). Synergistic effects between these mycotoxins have also been reported in in vivo studies with poultry, rodents and dogs (Koshinsky and Khachatourians 1994) as well as in in vitro studies. The co-treatment with OTA and CIT has been observed to increase the major DNA adduct formed by OTA (Pfohl-Leszkowicz et al. 2008). Co-administration of OTA and CIT was also seen to enhance the incidence of renal cell tumours in mice (Kanisawa 1984).

FB_1 (Gelderblom et al. 1992; Howard et al. 2001) and PA (Dickens and Jones 1961; Palmgren and Ciegler 1983) were also found to be carcinogenic mycotoxins and may interact with OTA, which is a potent carcinogen. Moreover, FB_1 was found to have a pronounced nephrotoxic effect on animal kidneys (Voss et al. 2001; Bucci et al. 1998), which can be additive to the nephrotoxic effect of OTA.

Some recent investigations on the interaction and cytotoxic effect of different combinations of OTA, PA, CIT and FB_1 on human peripheral blood mononuclear cells measured by MTT assay revealed additive or synergistic effects between OTA, CIT and FB_1 compared to any single mycotoxin (Stoev et al. 2009a). The absence of in vitro synergistic effect between OTA and PA can be explained by the specific mechanism of the in vivo synergistic effect of both mycotoxins (Stoev et al. 2001, 2004).

There is some new evidence that the toxicity of various strains of the same *A. ochraceus* group is completely different, depending on their capacity to produce both OTA and PA. A potent synergistic effect was seen between these mycotoxins, when the same were given simultaneously to pigs or chicks (Micco et al. 1991; Stoev et al. 1999, 2000a, 2001, 2004). It was found that contamination levels of OTA as low as 180 μg/kg, in combination with PA, can induce macroscopic kidney damage similar to spontaneous MPN after only 3 months of exposure (Stoev et al. 2001). The increase in OTA toxicity in these cases is shown to be due to the partially impaired detoxification of OTA by PA, when both mycotoxins are ingested simultaneously (Micco et al. 1991; Stoev et al. 1999, 2000a, 2001, 2004). Therefore, the multiple mycotoxin production by a single fungus, such as *A. ochraceus* (producing OTA and PA simultaneously), or by several fungi, seems to be a significant problem that has not been sufficiently investigated. Such mixtures of mycotoxins usually have synergistic or additive effects in farm animals, which could explain the potent toxic effect of comparatively low levels of OTA in the feeds for pigs (Stoev et al. 1998a,b), chickens (Stoev et al. 2002a) or humans (Stoev 1998).

Due to the potent toxic and synergistic effects between OTA and PA or CIT (Sansing et al. 1976; Stoev et al. 2001; Bernhoft et al. 2004) as well as between OTA and FB_1 (Klaric et al. 2007; Creppy et al. 2004), simultaneous exposure to those mixture of mycotoxins could be crucial for development of chronic renal failure in MPN. A second question then arises, whether such a mixture of mycotoxins is occurring in food and feed, and what are the contamination levels. So far, there is only scarce information about the contamination levels of PA and FB_1 in foods or feeds from areas endemic for MPN or BEN (Balkan Endemic Nephropathy). There are only a few reports that FB_1 and OTA co-occurred in maize in Croatia (Jurjevic et al. 1999, 2002; Domijan et al. 2005) as well as in feeds for pigs in Bulgaria (Stoev et al. 2009b), as the mean levels of FB_1 (459.8 and 5,564 μg/kg respectively) are not so low. It is important to mention that comparatively high contamination levels of OTA and FB_1 (up to 40 mg/kg) have been found in some pig feeds (Diaz et al. 2001) and were reported to provoke the death of the pigs, as there were pathological signs of both toxins, e.g. pulmonary oedema, liver and kidney lesions.

In addition, we should bear in mind that some rare and slightly nephrotoxic mycotoxins such as XA, CA and ERY can also have additional synergistic or additive nephrotoxic effects in addition to the mycotoxins already mentioned. The same mycotoxins were found in some of the feed samples from farms with MPN in South Africa, as can be seen from the present study, and also in Bulgaria (Stoev et al. 2009b).

Our results are also in good agreement with those of Miljkovic et al. (2003), who found that *P. polonicum*

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extract, which does not contain OTA or other known mycotoxins, when given to rats provoked significant and persistent pathomorphological changes in the nuclei of tubular epithelium in rats' kidneys, such as apoptosis and karyomegalic or mitotic changes, including formation of DNA adducts. This *P. polonicum* strain, which is a common food/feed spoilage mould in warm temperate areas such as South Africa, was also found to be a frequent contaminant in Bulgarian feeds suspected of causing spontaneous MPN (Mantle and McHugh 1993; Stoev et al. 1998a, 2009b). The same *P. polonicum* extract given to rats by Miljkovic et al. (2003) may also contain PA as the strains from *P. aurantiogriseum* group (including *P. polonicum*) are potent producers of PA. It was shown that PA can also provoke DNA breaks in mammalian cell lines as has been previously reported (Umeda et al. 1972). These changes (apoptosis and karyomegaly in tubular epithelium), provoked by *P. polonicum* extract, could also be induced by the new UM found in the present study, which needs to be further investigated. A recent experiment of Njobeh et al. (2009) suggests a potent cytotoxicity of the purified UM. This would be of interest, because the same mycotoxin could be partly responsible for the nephrotoxic damages described in South African nephropathy. On the other hand, the apoptotic changes provoked by UM could couple with apoptotic changes and DNA adducts provoked by OTA (Obrecht-Pflumio and Dirheimer 2000; Faucet et al. 2004). Such an interaction between OTA and other co-contaminants in commercial chicken/pig rations or human food would be very important and could explain the significance of the relatively lower doses of OTA exposure of chickens (Stoev et al. 2000a, 2002a), pigs (Stoev et al. 1998a,b) or humans (Stoev 1998). Particular attention has to be paid to the high incidences of these *Penicillium* spp., especially *P. polonicum*, which is the main fungus responsible for the high levels of this UM in animal or human feed/food. The other potential biological effects of the UM on mammalian cells should be also investigated in further in vivo or in vitro studies.

Together with *P. polonicum*, other *Penicillium* fungi such as *P. aurantiogriseum* and *P. commune*, were also found to be nephrotoxic to rats (Macgeorge and Mantle 1990) or to kidney tubule cells in tissue culture (Yeulet et al. 1988). Such fungi have been isolated from maize collected from BEN-endemic areas of former Yugoslavia as well as from MPN-endemic areas in Bulgaria (Stoev et al. 1998a, 2009b) and were supposed to produce an unknown biologically active glycopeptide fraction or secondary metabolite with cytotoxic properties. As can be seen in this study, we managed to identify such a substance (UM) from the same *Penicillium* fungi, isolated from areas with MPN in South Africa. The same substance was recently purified and studied for possible cytotoxic effect on human lymphocytes in comparison to other mycotoxins as OTA and T-2 toxin, and its toxicity at low concentrations (0.15 and 0.31 μg/ml) was found to be lower than toxicity of OTA and T-2, but at higher concentration (0.63 μg/ml) the toxicity of the same substance was seen to draw near to that of T-2 toxin (Njobeh et al. 2009).

Finally, we can conclude, that the synergism between various mycotoxins such as OTA, FB_1, PA, CIT and not yet chemically identified *P. polonicum* nephrotoxin in field conditions may be responsible for various spontaneous cases of MPN in South Africa and probably in some other countries. Having in mind the strong synergistic effects between OTA and PA or CIT (Stoev et al. 2001; Bernhoft et al. 2004) as well as between OTA and FB_1 (Klaric et al. 2007; Creppy et al. 2004), simultaneous exposure to those mycotoxins might be an important factor for development of renal diseases in South African pigs. It is therefore of great importance to investigate further the real toxic effect of combined administration of the same mycotoxins in in vivo studies using the same contamination levels found in the real practice.

Having in mind the different nature of MPN in various countries, we should realize that only an integrated approach to food safety that includes systematic identification and assessment of hazards in foods/feeds and various means to control them could resolve the current problems. Effective enforcement of food safety laws and regulations, in addition to further harmonisation of various national standards in regards to various mycotoxins and their combinations, are required to reduce the number of food-borne diseases and to enhance security of foods/feeds in order to protect the consumer and to ensure a global safety of various kinds of foods/feeds.

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